Signals for B cell activation in Antibody response

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Abstract

Germinal centres (GCs) are the sites where V-gene hypermutation and B cell selection are taking place. Testing specificity and affinity of GC B cell receptor by interaction with antigen on follicular dendritic cells (FDCs) may be an important selection process to select high affinity B cell clone. As antigen on FDC is present in the form of antigen-antibody immune complex, GC B cells are expected to have to compete with antibody to get access antigen. Initially this antibody will be of low affinity. However, during the course of an immune response, this affinity may increase.

We have tested this competitive selection model by following the replacement of antibodies in the GC over the course of an immune response. The speed of this replacement is dependent on affinity. Antibody added during an ongoing GC reaction can replace antibody in the GC, but only, if it is of high enough affinity. Presence of high or low affinity antibodies on FDC influences centrocyte selection, leading to variations in apoptosis within the GC, serum affinity, and plasma cell output. Parallel *in silico* experiments support the idea that a dynamic GC selection threshold, dependent on the affinity of GC output cells increases affinity maturation, because it enhances selection efficiency over a longer period during the course of a GC reaction. A dynamic selection threshold may explain the termination of the GC reaction, when affinity of new B cell variants is not sufficient to overcome the affinity of antibodies produced outside the GC.

IRF4 is essential for the plasma cell differentiation and Ig class switch. IRF4 mRNA and protein rapidly upregulate within one hour after naive B cells get stimulation with NP-Ficoll in QM×C57BL/6 mice, and then activated B blasts expressing intermediate level of IRF4 either go into the red pulp to form the early extrafollicular response by upregulating high level of IRF4, or travel into the follicle to differentiate into GC founding cells. IRF4 completely shuts down when

cells becomes proliferating centrocytes. But IRF4 expresses again in centorcytes, which have been committed to differentiate into the plasmablasts. Its high level expression shows the GC emigrants. Here IRF4 is selected as the marker for the early plasmablats appearance on the GC-T zone interface at the beginning of the GC reaction. And further experiments by using cytokines such as IL-6, IL-10, IL-21, costimulatory signals OX40, CD30 deficient mice show that these signals can affect the development of these early $IRF4^{+}$ plasmablasts on the GC-T zone interface in the TD antigen response.

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Common Abbreviations

AFC	Antibody forming cell
AID	Activation-induced cytidine deaminase
APC	Allophycocyanin
APRIL	A proliferation inducing ligand
β2m	beta-2 microglobulin
BAFF	B cell activating factor
BAFF-R	BAFF receptor
Bcl6	B cell lymphoma 6
BCMA	B cell maturation antigen
BCR	B cell receptor
Blimp-1	B-lymphocyte induced maturation protein-1
BrdU	5-Bromo 2′-deoxyuridine
BSA	Bovine serum albumin
bt	Biotin
C57Bl6	C57BL/6J
CGG	Chicken gamma globulin
CSR	Class switch recombination
DAB	3,3'-Diaminobenzidine tetrahydrochloride
DC	Dendritic cells
Dnky	Donkey
dKO	Double Knock out
DNA	Deoxyribonucleic acid
EBI2	Epstein-Barr virus–induced gene2
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunospecific assay
F	Follicle
FACS	Flow-assisted cytometric sorting
FCS	Fetal calf serum
FDC	Follicle dendritic cells
Fig	Figure
FITC	Fluorescein-isothiocyanate
GC	Germinal centre
Gt	Goat
Hs	Hamster
IC	Immune complex
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin

int.	Intermediate
i.p.	Intraperitoneal
IRR4	Interferon-regulatory factor 4
i.v.	Intravenous
J	Joining
κ	Kappa
λ	Lambda
KO	Knock out
LCM	laser capture microdissection
LFA-1	Lymphocyte function-associated antigen-1
LN	Lymph node
LPS	Lipopolysaccharide
LT	lymphotoxin
LTi cells	lymphoid tissue inducer cells
MFI	Median fluorescence intensity
mRNA	Messenger ribonucleic acid
MZ	Marginal zone
MS	Marginal sinus
NFκB	Nuclear factor of kappa light polypeptide gene enhancer in B-cells
NK	Natural Killer
NP	4-hydroxy-3-nitrophenyl acetyl
NP-CGG	4-hydroxy-3-nitrophenyl acetyl-Chicken Gamma Globulin
PALS	Periarteriolar lymphoid sheath
PAX5	Paired box gene 5
PBS	Phosphate buffered saline
PC	Plasma cell
PCR	Polymerase chain reaction
PE	Phycoerythrin
PKA	Protein kinase A
PNA	Peanut agglutinin
pNPP	p-Nitrophenyl Phosphate
Prdm1	Positive-regulatory-domain-containing
QM	Quasi-monoclonal
Rb	Rabbit
Rabbit PAP	Rabbit Peroxidase / Antiperoxidase complex
RNA	Ribonucleic acid
RPA	Replication protein A
Rt	Rat
RT2-PCR	Real time reverse transcriptase polymerase chain reaction assays
SDF-1	Stromal derived factor-1
Sh	Sheep

SHM	Somatic hypermutation
$S1P_1$	Sphingosine 1-phosphate receptor
SPR	Surface plasmon resonanace
SRBC	Sheep red blood cells
ST	Switch transcript
STAT	Signal transducers and activators of transcription
Strept AP	Steptavidin-Biotin Alkaline Phosphatase complex
T_{FH} cell	Follicle help T cell
TACI	Transmembrane activator and calcium modulator and cyclophilin ligand interactor
TCR	T-cell receptor
TD	T cell-dependent
TdT	terminal deoxynucleotide transferase
TH-1	T-helper type 1
TI-1	T cell-independent type 1
TI-2	T cell-independent type 2
TLR	Toll Like Receptor
TNF-	Tumour necrosis factor alpha
TNFR	Tumour necrosis factor receptor
Tris	Tris(hydroxymethyl)aminomethane
T zone	T cell zone or Periarteriolar lymphoid sheath
UNG	Uracil-N-NDA glycoslase
VCAM-1	Vascular cell adhesion molecule-1
VLA-4	Very late antigen-4
V-region	Variable region (of immunoglobulin)
Xbp-1	X-box binding protein 1

Table of Contents

List of Figure and Tables

Chapter One Introduction

1.1 Overview

Vaccination is designed to induce the rapid production of high affinity antibodies in response to infection. Antibody has two important protective functions. One is the opsonization of microorganisms leading to their destruction by phagocytes such as macrophages. Another is neutralization in which antibody blocks the molecular interactions required for toxins and viruses to enter the body's cells (Janeway CA 2005). Antibody responses derive from antigen-driven proliferation of B lymphocytes and their subsequent differentiation into antibody secreting cells. High affinity antibody responses develop via an immunoglobulin (Ig) hypermutation process that randomly generates antigen receptors of higher or lower affinity.

In T cell dependent antibody responses, germinal centres (GCs) are the sites where B cell proliferation, Ig V-gene hypermutation, and B cell selection are taking place (MacLennan 1994; Liu YJ 1997a). B cell maturation in GCs also involves Ig class switching (Liu YJ 1996b). This thesis is concerned with the development of antibody responses in lymphoid tissues, focusing on the identity and the role of signals during the germinal centre reaction.

Mutated GC B cells have to go through a Darwinian selection process in order to increase the affinity of their mutated B cell receptor. They test the specificity of their antigen-receptor by scanning over the surfaces of follicular dendritic cells (FDC), where antigen is held in the form of immune complex: complexes of antigen and antibodies formed during the response. We hypothesize that B cells receive positive selection signals during this interaction only if the affinity of their own antigen cell receptor can compete with the affinity of the antibodies in the immune complex. This may be a major mechanism, by

which high affinity antibody responses evolve. My first aim was to test whether antibody competition may act as a selection threshold mechanism for B cell selection in germinal centres. As antibody affinity will increase during the germinal centre reaction, higher affinity antibody will provide a positive feedback loop for the induction of high affinity GC selection.

IRF4, transcription factor, is essential for plasma cell differentiation and Ig class switch (Klein U 2006; Sciammas R 2006). In Chapter 4, IRF4 is used as a marker for the germinal centre selection and plasma cell output. This shows an early appearance of selected germinal centre emigrants at the GC-T zone interface. Furthermore, I am showing results on signals for the early exit of $IRF4^+$ plasmablasts from germinal centres. For this, mice deficient for cytokines and costimulatory molecules possibly involved in germinal centre B cell selection are used to follow plasmablasts exiting from germinal centres. With a combination of histology, microdissection and RT-PCR I am trying to define these signals and locate the site of their production.

1.2 The structure and function of murine spleen

Lymphoid organs are organized, compartmentalized cellular structures. In these organs, interactions between lymphocytes and with non-lymphoid cells are important for lymphocyte development, initiation of the adaptive immune responses, and their survival maintenance. The primary lymphoid organs are the bone marrow where B lymphocytes mature, and the thymus where precursor T lymphocytes originating in bone marrow undergo their maturation (Janeway CA 2005). Secondary lymphoid organs (such as the lymph nodes and spleen) and mucosal-associated lymphoid tissues are discrete sites where lymphocytes are maintained and encounter antigens to generate adaptive responses. Immature lymphocytes leave primary lymphoid organs where they mature in secondary lymphoid tissues. Mature lymphocytes recirculate through the blood and secondary

lymphoid tissues where foreign antigen and antigen-presenting cells can efficiently concentrate and activate a small number of antigen specific lymphocytes during an immune response (Janeway CA 2005; Bergstrom CT 2006)

This thesis investigates the signals for B cell differentiation during the development of high affinity antibody responses in murine spleens. The spleen is central in the defence against blood-borne immunogens, such as foreign antigens, old or damaged cells, and contributes most significantly to defence against bacterial pathogesn (e.g. *Salmonella* (Brendolan A 2007) (Fig 1.1). In essence, the spleen is comprised of two functionally and morphologically distinct compartments: the red pulp and the white pulp (Janeway CA 2005). The red pulp consists of a three dimensional meshwork of splenic cords and venous sinuses. The splenic cords compose fibroblasts, reticular fibers, and are associated with macrophages. This specialized structure provides an area with the unique capacity to filter blood and remove foreign material and old erythrocytes. The spleniccords in the murine red pulp are associated with are haematopoietic cells (Johnson 1975), lymphocytes, as well as plasma cells and plasmablasts that migrate from the follicle and outer T zones after antigen induced activation (Wolber FM 2002; Mebius RE 2005). Plasmablasts are attracted to the extrafollicular foci. Within the red pulp, it has been found that $CD11c^{high}$ dendritic cells (DCs) which are associated with plasmablasts have a role in plasma cell survival (de Vinuesa CG 1999; Vinuesa CG 2000). In addition, the red pulp contains poorly-characterized niches that may sustain plasma cell survival (Sze DM 2000).

Bridging channels are less defined, at moment known as small areas where the follicles surround the T zone, enabling the T zone to makes direct contact with the red pulp. Bridging channels are the exits for recirculating B cells and T cells from the white pulp (Bajenoff M 2008) and also an exit point for plasmablasts generated in extrafollicular foci

in the adjacent red pulp (Jacob J 1991; Liu YJ 1991; Toellner KM 1996). Antibody forming cells temporarily reside in these channels after antigen challenge (Leenen PJ 1998).

1.2.1 T zone

The T zone lies at the centre of each white pulp, surrounding the central arteriole. It largely contains T cells, but also comprises interdigitating DCs, and B cells after activation. The boundary of the T zone and the follicle (also called the outer T zone) is the site of interaction between activated B cells with primed T cells (Toellner KM 1996; Garside P 1998). Recirculating T cells in the blood travel though the marginal zone (MZ) into the T zone, and then test their T cell receptor (TCR) against the antigen presented in the context of MHC class I or II molecules or CD1d by DCs, this initiates the process of T cell priming (Austyn JM 1988; Stoll S 2002; Mempel TR 2004). These T cells enter cell cycle and then migrate to the outer T zone by co-expressing CCR7 and CXCR5 (Hardtke S 2005). They interact with primed B cells that have taken up and processed antigen via their BCR at the boundary of the T zone and the follicle (Toellner KM 1996; Okada T 2005). B cells are induced to migrate to these areas in both TI and TD responses (de Vinuesa CG 1999b; Okada T 2005; Hsu MC 2006)

1.2.2. The follicles

The follicles (B cell areas) surrounding the T zone are found at bifurcation sites of the central arterioles (Ward JM 1999). They are predominantly composed of recirculating B cells with few follicular dendritic cells (FDC) and $CD4^+$ T cells (van Rees 1996; Tew JG 1997). Characteristically, recirculating B cells are IgD^{high}, IgM^+, $CD23^{high}$, $CD21^+$, $B220^{high}$ (Coffman RL 1981; Gray D 1984; Grey HM 1984; Ling NR 1987). They are found throughout primary follicles and are in a constant state of migration between the

follicles of secondary lymphoid tissues (Nieuwenhuis P 1976). Primary follicles contain naïve recirculating B cells, the FDC network, and no germinal centres (GC) whilst secondary follicles contain GCs, which form upon antigenic stimulation. Germinal centres contain activated GC B cells, FDCs, tingible body macrophages, T cells and apoptotic B cells. GCs are the sites of somatic hypermutation and Ig class switching (MacLennan 1994).

Recent evidence indicates that in lymph nodes recirculating B cells (naive B cells) can take up antigen from macrophages and FDCs in the first few days following antigen administration (Phan TG 2007; Suzuki K 2009). FDCs have been considered to be responsible for antigen presentation to B cells in secondary lymphoid organs. They can retain the extracellular antigen in a plasma membrane-associated form in the follicles for months after immunization (Nossal G 1968; Batista FD 2009). Furthermore, it is known that FDCs in follicles can mediate the retention of antigen in the form of immune complex (Mandel T 1980; Tew JG 1980) through complement receptors (Chen 1978; Tew JG 1997) and Fc receptors (Yoshida 1993; Qin D 2000). The special arrangement of immune complex on FDCs has been found to promote B cell somatic hypermutation *in vitro* and *vivo* (Song H 1998; Sukumar 2008; Wu Y 2008). Also, FDC are considered to be potent accessory cells during B cell activation, possibly secreting chemokines and/or FcγRIIB (Tew JG 1980; Tew JG 2001). The detail about the role of FDCs derived immune complex on somatic hypermutation and antibody production will be further discussed in Chapter 3.

The importance of $CD3^{-}CD4^{+}$ adult lymphoid tissue inducer cells (LTi cells) in organising the development and perhaps the maintenance of secondary lymphoid organs has been highlighted (Mebius 2003; Lane PJ 2005). Signals from LTi cells are sufficient to regulate T cell organisation and the expression of podoplanin and CCL21 on T zone

stoma (Withers DR 2007). Furthermore, adult LTi cells may have a role for high quality memory B cell responses ((Lane PJ 2005) and papers from Lane group). Adult LTi cells associate closely with $VACM^+$ FDCs in follicles where they foster the survival of follicular T helper cells in GCs, as well as with the $VACM^+$ stroma population within the B-T zone interface where LTi cells foster the survival of memory T cells (Kim MY 2007).

1.2.3. The marginal zone

The marginal zone (MZ) is a unique region of the spleen that is situated at the interface of the red pulp and white pulp (the T zone and B cell follicles) (Cesta 2006). The MZ contains reticular fibroblasts, B cells, marginal zone macrophages and dendritic cells (Martin F 2002; Mebius RE 2005; Cesta 2006). Marginal zone macrophages form the outer ring of macrophages, characterised by expression of the C-type lectin SIGNR1, the receptor for blood-borne antigen (Geijtenbeek 2002; Kang YS 2003; Karlsson MC 2003), and the type 1 scavenger receptor MARCO that is important for the uptake of various bacteria (Elomaa O 1995). Marginal zone metallophilic macrophages form an inner ring of the marginal zone adjacent to the follicles and T zone (Munday J 1999). The inner limit of the MZ blood sinusoids and peripheral to marginal zone metallophilic macrophages is the marginal sinus (MS). It is continuous with vessels that feed the capillary beds of the white pulp (Mebius RE 2005; Cesta 2006). The cells in the MZ come into contact with the blood from the MS (Schmidt EE 1988). Therefore, the MZ is considered to screen the systemic circulation for antigens and pathogens, having an important role in antigen processing. The MS is the transit area at which both recirculating T and B cells leave the bloodstream and enter the white pulp (Mebius RE 2005) (Fig 1.1). MZ B cells are a unique subset, and are distinct from follicular B cell (discussed in detail in section 1.3.3).

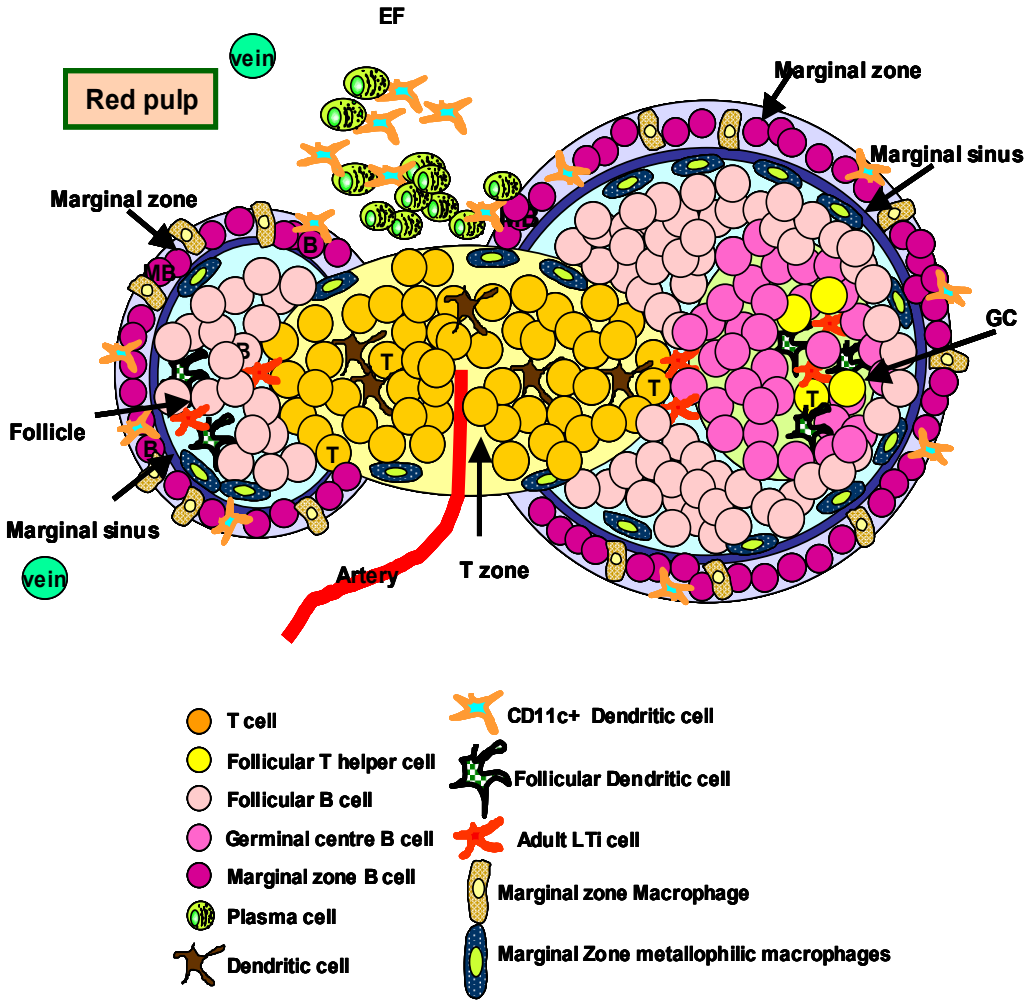

Figure 1.1: The structure of the white pulp of the murine spleen
The murine white pulp mainly consists of the T zone and the B cell rich follicles. T zones are surrounded by follicles. The follicles are separated from the marginal zone (MZ) by the marginal sinus (MS). Extra follicular plasma cell foci (EF) are formed at the bridging channels where the red pulp abuts the T zone early during antibody responses. Germinal centres (GC) form in the follicle after antigen stimulation.

1.3 B cell subsets

B cells develop from pluripotent haemopoietic stem cells in the liver during mid-to-late fetal development, and in the bone marrow after birth. They can provide humoral immunity by producing soluble immunoglobulin against free antigen in solution (Stites DP 1994). B cells are divided into distinct subsets: B-1, recirculating B2 and MZ B cells. Additionally, in the mouse B-1 cells are subdivided into B-1a which express CD5, so called $CD5^+$ B cells, and B-1b, which do not express CD5. B-1 are a minority subset of B cells comprising about 5 % of all B cells in adult mice and humans (Janeway CA 2005). B-1a, B-1b, and recirculating B2 cells undergo distinct developmental pathways. (Montecino-Rodriguez E 2006; Tung JW 2006), and MZ B cells can originate from all three kinds of cells. However, all B cells follow a common initial developmental pathway, originating from haematopoietic B cell precursors (Hardy RR 2001; Tung JW 2006). This thesis mainly focuses on recirculating B2 cells, also called recirculating follicular B cells.

1.3.1 Recirculating B2 cells and their development

B2 cells represent the major B cell population in the body. Their Ig repertoire is diverse with random VDJ segment usage and extensive N-additions (Feeney 1990). During adult life there is a continued low level input of newly formed B cells into the recirculating pool. Recirculating B2 cells have a limited capacity to respond to TI antigens (Lane PJ 1986). The rearrangement of V, D, and J Ig genes is a fate decision step for developing B cell in the bone marrow, which is initiated during the intermediate pro-B stage, termed antigen independent diversification. This, unlike class switching, is not dependent on antigen activation. The rearrangement process is not analogous to that of Ig class switch recombination (CSR), however, both involve somatic DNA recombination to improve the antibody repertoire.

Once a functional and fully formed IgM molecule composed of a successfully rearranged H and L chain, is expressed on the cell, the cell immediately becomes an immature B cell, whilst highly self-reacting newly-formed B cells are induced to die. However, some immature B cells which have autoreactive BCR can be eliminated to become tolerant or anergic (Rolink A 1994). Immature B cells leave the bone marrow to undergo further differentiation to become mature B cells that co-express IgD, which is accomplished by alternative mRNA splicing (Janeway CA 2005).

Immature B cells can enter the circulation and then T zone of the spleen (MacLennan IC 1986). A proportion of these cells are selected either to mature and become recirculating B2 cells that enter the follicles, or to become MZ B cells (Bazin H 1987). The precise mechanism by which this selection occurs is only partially understood, but it appears to involve a process that at least in part, BCR specificity (Cariappa A 2002), non-specific factors, such as B cell-activating factor (BAFF), are also necessary for B2 cell maturation and survival (Schiemann B 2001). Some polyreactivity, including self-reactivity of BCR may drive B cells to become MZ B cells or B-1 cells (Kanayama N 2005; Xing Y 2009). Most of the immature B cells (about 90%) die within 1-3 days upon arrival in the spleen if they are not selected to mature in the periphery (Chan EY 1993). While the life span of mature recirculating B2 cells is five to six weeks (Calame KL 2003), the life span of MZ B cells is probably in the range of months (Carvalho TL 2001).

1.3.2 B-1 cells

The B-1 pool is largely found in the peritoneal, pleural cavities and lamina propria, with only a few B-1a cells residing in the spleen and lymph nodes (Hardy RR 1994; Hsu MC 2006). They mainly derive from foetal liver and omentum (i.e. B-1a) in the mouse, although small number of cells can be produced in adult life (Wortis HH 2001). The B-1

cells do not, or express a low levels of CD45RA compared to B2 cells which express higher level (Montecino-Rodriguez E 2006). In addition, the phenotype of B-1 cells shares some phenotypic features with that of MZ B cells; they are both $IgM^{hi}IgD^{low/-}$ and $CD21^{+}CD23^{low/-}$ (Janeway CA 2005). Mouse peritoneal B-1 cells additionally express CD11b, which is down-regulated in splenic B-1 cells (Martin F 2001b). Within this population, cells not expressing CD5 are referred to as B1b cells as opposed to $CD5^{+}$ B-1a cells (Hardy RR 2001).

B-1 cells, unlike B2 cells arise from a development pathway independent of IL-7 signalling but dependent on CD19 expression (Carvalho TL 2001). The possible reason is that in mature mice B-1 cell numbers are maintained by self-renewal by ubiquitous self and environmental antigens, rather than from haemopoiesis. In addition, Esplin (2009) found that B1 cells could be derived from both early lymphoid and common lymphoid progenitors (Esplin BL 2009). The expression of CD19 affects the fate of B-1a and B-1b cells. For example, overexpression of CD19 during mouse development produces more B1a than B1b cells, and the opposite effect is observed in CD19 knock-out mice (Haas KM 2005). CD19 and CD21 are important in synergising the BCR signal transduction, whilst CD5 may inhibit BCR signalling via SHP-1 (Sen G 1999).

B-1 cells have a low threshold of activation compared to B2 cells (Fagarasan S 2000). They can form plasmablasts in extrafollicular foci, and differentiate into plasma cells in the spleen (Hsu MC 2006) omentum (Ha JP 2006) and lamina propria of the gut (Rosado MM 2009). B-1 cells can produce natural antibody (Hardy RR 2001), largely composed of IgM or secretory IgA. They may promote TD contact sensitivity responses when they move into the spleen within 24 hours to secrete IgM (Itakura A 2005). B-1a cells can respond to TI antigens (i.e. polysaccharide antigens, NP-Ficoll) (Hsu MC 2006), which engage their BCR. Recently they were found to be responsible for the early response to

Salmonella Typhimurium (Gil-Cruz C 2009).

The BCR repertoire of B-1 cells is limited, with no N-nucleotide additions in V_H-D or D-J_H joints, possibly because B-1 cells develop early (when TdT is not expressed). Consequently, this restricts the range of V_H regions used and antigen-binding specificities (Feeney A J 2000). Therefore B-1 cells carry out a more primitive, less adaptive, immune response than conventional B2 cells.

1.3.3 Marginal Zone B cells

Marginal zone B cells usually reside in the marginal zone of the white pulp. They appear to be resting mature B cells but they express different surface proteins from follicular B cells. Phenotypically they are characterised by the following expression profile: $IgM^{high}IgD^{low/-}$ and $CD21^{high}CD23^{low/-}$, and do not express CD5 or CD11b in mice, unlike B1a cells (Balazs M 2002; Hsu MC 2006). However, some MZ cells are switched and do not express IgM or IgD (Pape KA 2003).

Margnal zone B cells have several origins: immature B2 cells can be recruited directly into the MZ B cell pool (Balazs M 2002), and recirculating B cells can be recruited to become MZ B cells (Kumararatne DS 1981; Vinuesa CG 2003). B-1 cells might also be recruited into the MZ pool (Martin F 2000; Pillai S 2005). Furthermore, memory B cells colonize the MZ B cell population (Liu YJ 1988; Toellner KM 1996) and are known as MZ memory B cells, which are largely derived from the GC (Dunn-Walters DK 1995).

Marginal zone B cells are ideally located to respond blood-borne pathogens. They leave the MZ stroma on activation. Both TD and TI-2 antigen activation can promote their movement into the out T zone (Liu YJ 1988; Vinuesa CG 2001). MZ B cells actively

transport antigen-antibody complexes to FDCs via their CR1/CR2 receptors (Gray D 1984; Molina H 1996). In addition, activation with LPS (Pettersen JC 1967) or via their Fc receptors (Brown JC 1970) induces the migration of MZ B cells to follicles.

Marginal zone B cells continuously shuttle back and forth between the MZ and the follicles (Cinamon G 2008). The movement of MZ B cells to the follicle is mainly dependent upon the up-regulation of CXCR5 which enables migration towards CXCL13, produced by FDCs in the follicles, whereas return to the marginal zone requires the sphingosine 1-phosphate receptor ($S1P_1$) and to some extent $S1P_3$ present in the blood in the MZ sinusoids (Lu TT 2002; Cinamon G 2004; Cinamon G 2008).

Marginal zone B cells are very easily activated (Oliver AM 1997), and in particular are rapidly recruited into both TD and TI extrafollicular antibody responses (Liu YJ 1991; Toellner KM 1996). They can produce natural antibodies together with B1 cells as the main source of natural antibody (Pillai S 2005). Their high expression of complement coreceptor CD21 enables them to respond actively to TI-2 antigens (Zandvoort A 2002), some of which fix complement and bind C3d without the requirement for prior interaction with soluble Ig. They also have the capacity to form GCs (Song H 2003).

1.4 B cell activation

1.4.1 T cell dependent activation

Most of B cell responses considered are dependent upon T cell help and are thus known as thymus dependent (TD) antibody responses. There are two separate stages in the process by which T cells help B cells to make antibody. The first step requires physical contact between the B and T cell, and specific recognition of peptide presented by the B

cell in association with its class II MHC molecules, and co-stimulation via B7.1 or B7.2 molecules on B cells and CD28 on T cells. Once activated through physical interaction with a T cell, B cells can respond in a non-cognate fashion to molecules that are produced and released from T cells or other cells (Janeway CA 2005).

Naïve T cells are unable to provide help for B cells until they become primed by antigen presenting cells, including B cells or dendritic cells (DCs). The later are found in the T cell rich areas of secondary lymphoid organs. *In vivo* studies demonstrated that antigen-charged B cells focus on interacting with T cells activated by DCs (Toellner KM 1998). It takes at least 2 days before sufficient numbers of primed T cells are available in T zone (PALS) to activate B cells (Hodgkin PD 1996).

When the primed T cell recognizes antigen presented by the B cell, it provides signals such as OX40 and CD40-Ligand that result in the local growth of both cell populations (MacLennan IC 2003). By day 4 after immunization, activated B cells proliferate and differentiate along two divergent pathways (Shih TA 2002a). After two cell cycles, B cells are induced to become plasmablasts, migrating from the out T zone to the local site of extrafollicular growth (the extrafollicular antibody response): the red pulp of the spleen, and the medullary cords in lymph nodes, and they then differentiation into early plasma cells which characteristically produce non-mutated low affinity antibody. Expression of the chemokine receptor CXCR4 by plasmablasts may be responsible for the migration of plasmablasts to extrafollicular foci (MacLennan IC 2003). Plasmablasts proliferate extensively for 3 days in extrafollicular foci before they differentiate into plasma cells (Sze DM 2000). Non-dividing plasma cells synthesize and secrete large amounts of Ig. Most plasma cells lost from the spleen after 2-3 days and only a minority of cells surviving as long-lived plasma cells (Smith KG 1996; Sze DM 2000). The other activated B cells travel to follicles and begin proliferating to form germinal centres by day 7 after

primary immunization (known as the follicular response) (Toellner KM 1996). (The basic pathways of B cell migration after stimulation see Fig 1.2)

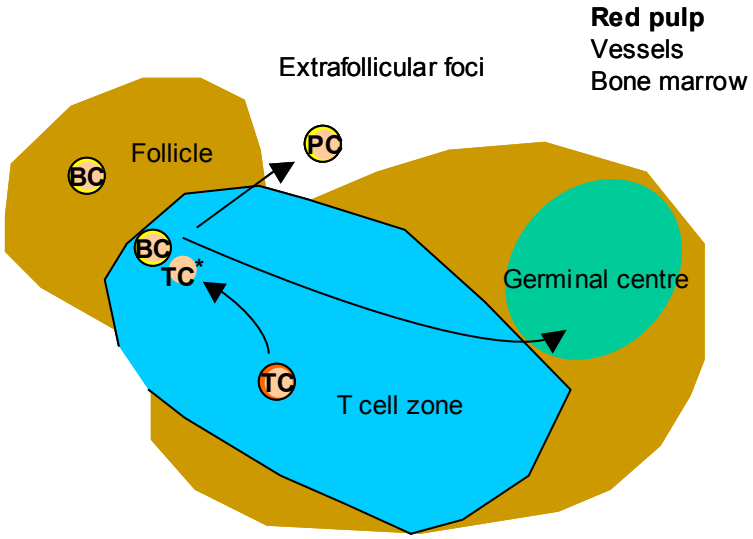

Figure 1.2: B cell activation
The primed T cells help B cell activation, and then activated B cells travel to the outer T zone, some differentiate into the early plasmablasts in extrafollicular foci, and others go to the follicle to form germinal centres (BC: B cell, TC: T cell, PC: Plasma cell) (Taken from Dr. K-M. Toellner)

Mice that are primed with antigen and are then challenged with the same antigen some weeks later undergo a secondary immune response. The onset of B cell proliferation in this response is shortened to less than 12 hours, as T cell help is readily available due to memory through previous antigen encounter (Toellner KM 1996; Sze DM 2000). The extrafollicular plasmablast response is seen between 2 and 3 days when those B cells that have moved into the red pulp begin to divide exponentially. By day 4 most of these cells have left cell cycle and become terminally differentiated plasma cells. GCs are seen by day 3 after immunization, but these are smaller than those formed during the primary response (Toellner KM 1996). This is probably due to the fact that memory B cells are more likely to differentiate into plasma cells, whilst naïve B cells prefer the germinal centre pathway (Arpin C 1997).

1.4.2 T cell independent activation

B cells can be activated by some antigens without T cell help. These antigens are characterized by their ability to induce immune responses in nude, athymic mice (Vinuesa CG 2000) and fall into two main classes: T independent type 1 (TI-1) antigens such as lipopolysaccharide (LPS) which is associated with the outer layer of the cell membrane of Gram-negative bacteria. LPS can stimulate responses not only in mature B cells but also in neonatal B cells. TI-1 responses exhibit little evidence of memory. These antigens are recognized by innate B cell surface receptors such as TLR4 and CD21 that can deliver costimulatory signals to B cells (Shih TA 2002b).

TI-2 antigens possess characteristics embodied by polysaccharide antigens. These antigens cannot evoke antibody responses in humans until several months after birth, and full adult responses to them do not develop until 5 years old (Murray CJ 1997). They do not elicit strong memory responses (Mond JJ 1995), although some development of memory B cell-like cells has been described (Obukhanych TV 2006). Due to their presence of repeating epitopes, these antigens have the capacity to induce a substantial amount of surface Ig cross-linking between B cells that provides a strong signal of activation (Vinuesa CG 2001).

1.4.3 Haptens

Hapten molecules have been used to study antigen and their interaction with antibody, because they are small defined structures that are good B cell antigens. They are unable to induce antibody responses on their own, and have to be conjugated to immunogenic proteins. Carrier proteins (such as Chicken Gamma Globulin, CGG) can induce T cell help, resulting in the induction of hapten specific antibody responses. The same haptens can also be conjugated to TI carriers (e.g. Ficoll) making it possible to study TI responses.

Therefore, it is possible to produce transgenic mice that have hapten-specific BCR to both types of responses (Janeway CA 2005).

The hapten 4-hydroxy-3-nitrophenol (NP) conjugated to a variety of carriers has been used for several decades to provoke a restricted immune response in mice. It has the attraction of inducing a canonical response which is highly biased towards Igλ light chain and the IgH chain V segment-V186.2-DFL16.1-Jh2 (Bothwell AL 1981). Therefore, it has been used to study somatic hypermutation (SHM) and affinity maturation when it is conjugated as immunogens such as NP-CGG and NP-Ficoll (Bothwell AL 1981; Azuma T 1987; Maizels N 1988).

1.5 B cell responses in Germinal Centres

1.5.1 T cell dependent immunization

Within 3 days of immunization naïve T cells are primed on interdigitating dendritic cells in the T zone (Luther SA 1997; Toellner KM 1998). B cells that have interacted with primed T cells in the outer T zone migrate into follicles, where they form GCs, which are a hallmark of the TD adaptive immune response. In GCs B cells proliferate and undergo SHM of their Ig genes, after which mutant clones that have acquired increased affinity for antigen (affinity maturation) are selected. These high-affinity cells form the progenitors of both the long-lived antibody-secreting plasma cells and the memory B cells that sustain serological immunity after infection (MacLennan 1994).

As the GC expands non-responding IgD^+ B cells are displaced and form the follicular mantle. B blasts proliferate and within about 4 days the GC reaches peak size (Liu YJ 1991). During this time the mature GC segregates into distinct dark and light zones (Liu YJ 1992; Camacho SA 1998). Earlier studies showed that molecules such as chemokines

CXCR4 and CXCR5 are very important for GC organization (Allen CD 2004), however, the details of GC organization still remain unclear. The GC light zone can be differentiated into the inner and the outer light zone. While the inner light zone is abundant in FDCs, the outer light zone, which is adjacent to the follicular mantle, is rich in T helper cells (MacLennan 1994).

The dark zone (DZ) contains the bulk of proliferating centroblasts. Centroblasts were named for their appearance as large mitotically active cells that lack surface Ig. The prevailing view is that SHM and class switch recombination takes place in the DZ (MacLennan IC 2003). This represents the first step in affinity maturation, and since base changes are introduced stochastically, it results in Ig with a wide range of affinities for the immunizing antigens. The light zone (LZ) is located distal to the T cell-rich area adjacent to the primary follicle. This is where FDCs, T cells and centrocytes differentiated from centroblasts, are located. FDCs have the unique capacity to present antigen to centrocytes in the form of antigen-antibody complexes containing various Ig isotypes with different affinities (Kosco-Vilbois 2003).

Antigen in immune complex that is bound by antigen-specific GC B cells may be taken up, processed and presented to antigen-specific follicular helper T cells (T_{FH}) to raise T cell help (MacLennan 1994; Liu YJ 1996a). Centrocytes test their new surface Ig antigen receptors against antigen displayed on FDCs. Interaction with T_{FH} may lead to migration back to the DZ which may result in further proliferation, SHM and selection, leading to a further improvement of antigen receptor affinity (MacLennan 1994; Liu YJ 1997a; Camacho SA 1998). Finally centrocytes expressing high-affinity and non-self-reactive antibody may be positively selected to differentiate into long-lived plasma cells or memory B cells. This most likely occurs in the LZ (Cattoretti G 2006a). By contrast, low affinity or self reactive centrocytes undergo apoptosis, either because they are not

stimulated through their B cell receptors, or because they do not find self-antigen specific T cells to interact with (Shokat KM 1995; Shih TA 2002a). Thus centrocyte selection mediated by T_{FH} cells within GC provides the basis for affinity maturation of the antibody response and B cell memory formation. The signals that initiate centrocyte differentiation into either memory cells or plasma cells are poorly understood.

1.5.1.1 Somatic Hypermutation and Class switch recombination

Centroblasts undergo SHM of their BCR V, D, and J genes in the GC, which leads to refinement and changes in the specificity of the humoral immune response. SHM is a mutational process used to create additional diversity after functional Ig genes have been assembled. High rates of point mutations are randomly introduced into the H and L chain V-region exon, leading to either an increase or decrease in the affinity of the resulting Ig for its target antigen, known as "affinity maturation" (Stites DP 1994; Jacobs H 2001; Kenter AL 2004). Activation-induced cytidine deaminase (AID) is the first of a complex series of proteins that initiate SHM of the antibody V region that encodes the antigen-binding site (Review on (Peled JU 2008)).

Class switch recombination (CSR) is the mechanism by which the IgV regions are recombined with different heavy chain genes, to produce other classes beyond IgM. This alters the effector function of the antibody, but not the specificity. CSR can be activated by T cell dependent signals, i.e. CD40 ligation, together with T cell derived cytokine signals. For example, in mice, CD40 ligation with IL-4 production, characteristic of Th2 responses, classically induces switching to IgG1 and IgE. In contrast CD40 ligation with IFNγ (Th1 response) is associated with switching to IgG_{2a} (Toellner KM 1998). CSR can also occur in TI extrafollicular responses. For instance, TI-1 antigens in mice induce mainly IgG_{2b} production, while TI-2 antigens induce switching predominantly to IgG3 (Perlmutter RM 1978).

Recent studies demonstrated that commonly CSR results from the fusion of an upstream switch (S) region to a downstream S region with the deletion of intervening DNA on heavy chains (Fig1.3). Recombination of DNA occurs in the switch region and depends on the inducer (I) exon, a small exon upstream of the switch region, and the switch region that precedes the heavy chain constant region (Harriman GR 1996; Manis JP 2002). Transcription from the I promoter can result in the production of a sterile germline heavy chain transcript (Stavnezer-Nordgren J 1986). CSR will only occur if this transcript is produced and spliced (Lorenz M 1995; Hein K 1998).

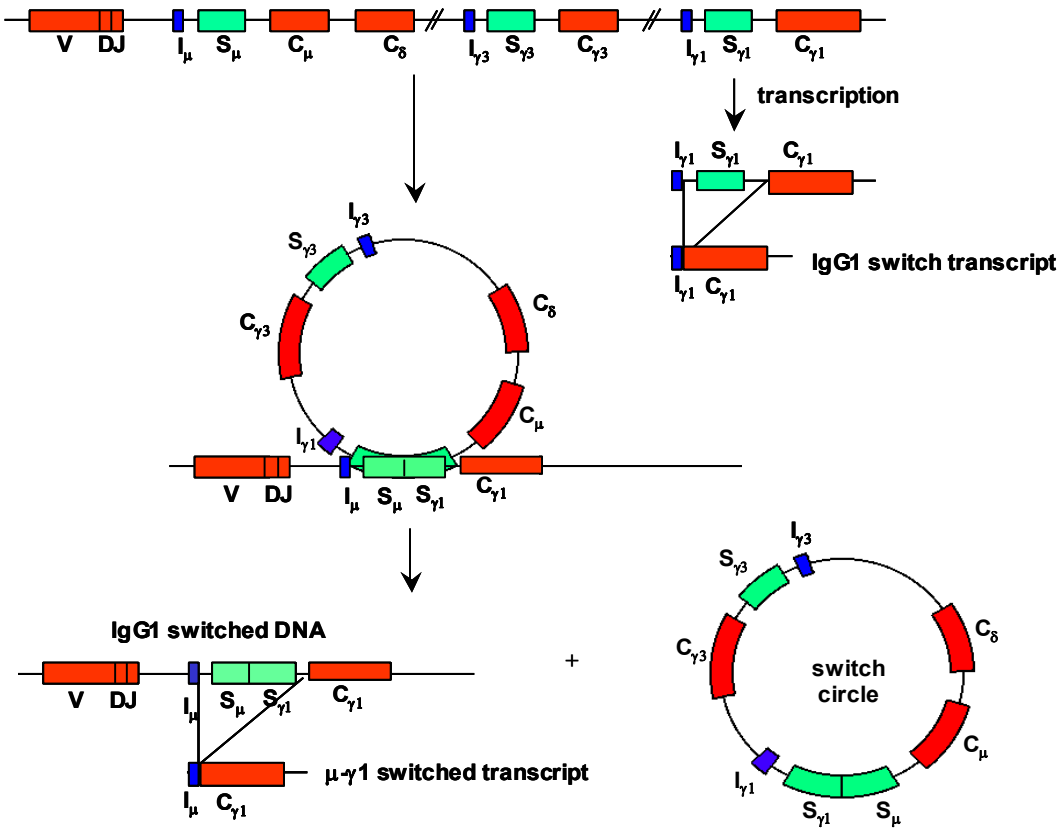

Figure 1.3: The mechanism of CSR illustrated by the switching to IgG1
Transcription occurs from Iγ1 locus and a germline switch transcript is produced. This is spliced and switching occurs via the looping out of the intervening DNA. This results in a switch circle and a rearranged heavy chain transcript. (Taken from Dr. K-M Toellner)

AID is also required in this process, this enzyme converts cytosines in both S regions, inducing breaks in the DNA. This allow the excision of the intervening regions and the production of a switch circle (Schrader CE 2005). As a consequence of this process, a U:G mismatch in the Ig locus may be repaired through uracil removal by uracil-N-glycosylase (UNG) or by base-excision repair and mismatch repair (de Yebenes VG 2006). Mutations in the AID gene lead to deficits in CSR, SHM and gene conversion of IgV genes (Muramatsu M 2000; Kenter 2005). Mice deficient in AID have a form of hyper IgM syndrome type 2, characterized by the presence of large TD GC, but with minimal hypermutation and class switching (Revy P 2000). AID expression is restricted to GC B cells, and its expression and activity are highly regulated even though it is unclear as to how AID is targeted to the Ig locus (Review on (Stavnezer J 2008). For example, miRNA 155 has been shown to downregulate AID expression (Dorsett Y 2008; Teng G 2008) whilst miR-181b could inhibit AID expression and subsequently CSR to γ1 (de Yebenes VG 2008). IL-4 and anti-CD40 stimulation could activate AID expression, possibly through binding signal transducer and activator of transcription (STAT6) and nuclear factor kappa light polypeptide gene enhancer in B-cells (NF-κB) to the 5' promoter region (Dedeoglu F 2004). Transcription factors including E proteins (Sayegh CE 2003), Pax5 (Gonda H 2003), IRF8 (Lee CH 2006) and IRF4 (Sciammas R 2006) may have a role for AID expression. Furthermore, the activity of AID requires replication protein A (RPA) (Chaudhuri J 2003) and phosphorylation by protein kinase A (PKA) (Basu U 2005; Pasqualucci L 2006).

1.5.2 TI immunization resulting in germinal centre formation

We have previously observed that GCs can also be induced by the TI-2 antigen NP-Ficoll, when high frequencies of B cells contain an antigen-specific B cell receptor, for example in quasimonoclonal (QM) mice (Vinuesa CG 2000). Extrafollicular foci develop within 3

days as in a wild-type the response to NP-CGG. GCs are easily detected 3 days after immunization, with the similar kinetics as in the secondary TD immune responses (Vinuesa CG 2000; Lentz VM 2001; Toellner KM 2002). But unlike the GC induced by TD antigen, TI GC does not contain T cells and spontaneously abort by 5 days post-immunization. Extrafollicular plasma cells and GC cells produced have been found to undergo a very limited mutation, but significant amount of Ig V-region recombination. The predominant antibodies produced are IgM and IgG3 (Toellner KM 2002). This also suggested that GC B cell survival, selection and differentiation all require T cell help. Germinal centre B cells in the TI response, like their TD counterparts also express the known GC B cell markers such as PNA, GL-7, Fas, and have downregulated IgD (Vinuesa CG 2002).

1.5.3 B cell lymphoma 6 (Bcl-6)

The Bcl-6 proto-oncogene encodes a nuclear transcriptional repressor, with pivotal roles in the GC formation as well as the regulation of lymphocyte function, differentiation, and survival (Jardin F 2007). Bcl6-deficient mice display normal B cell development but lack germinal centre development and affinity maturation (Dent AL 1997; Ye BH 1997). The Bcl-6 is protein highly expressed on germinal centre B cells. Naïve B cells also contain Bcl-6 mRNA and but do not produce detectable amounts of the protein, indicating that Bcl-6 expression is regulated at the post-transcriptional or post-translational level (Allman D 1996).

Bcl-6 has some important functions for GC B cells, including promoting cell cycle progression and inhibiting differentiation (Tarlinton D 2008b). Firstly, Bcl-6 suppresses apoptosis and cell cycle arrest via suppression of the p53 and p21, associating with N-CoR SMRT or B-CoR together to transcriptionally repress of genes (i.e. ATR). This

suppression could enable GC B cells to undergo vigorous proliferation while undergoing genetic remodelling by SHM and CSR without eliciting the growth-arrest and apoptotic responses seen in other cell types (Ranuncolo SM 2007; Ranuncolo SM 2007). Secondly, Bcl-6 inhibits the expression of genes involved in B cell activation such as CD69, CD44, STAT1 (Shaffer AL 2000) and CD80 (Niu H 2003). This serves to prevent premature activation of B cells before they have completed proliferation and SHM. Thirdly, Bcl-6 may inhibit the differentiation of GC B cells into plasma cells (Falini B 2000; Basso K 2004) and differentiation into memory B cells (Kuo TC 2007).

1.5.4 Migration within the Germinal Centre

The dynamic model of GC B cell development described above has been termed the "Cyclic-re-entry" model (Brink 2007). Recently, Haberman *et al* (2003) proposed a dark zone selection model in which recognition of soluble antigen within the DZ may permit B cell selection and proliferation, and movement into the LZ resulting in B cell differentiation (Haberman AM 2003). Some cell types (e.g. proliferating and apoptotic B cells and tingible-body macrophages) proposed to be required for selection are accessible in the DZ, at either the DZ periphery or at the follicle-T zone interface (Camacho SA 1998; Haberman AM 2003; Hauser AE 2007a). In mature GCs, portions of the DZ base are in direct contact with the adjacent T zone which contains activated T cells and DCs during the TD response (Grouard G 1996; Pape KA 2003; Okada T 2005). Houser *et al* (2007b) suggests that DCs in the T-B zone interface are theoretically capable of providing some signals similar to those thought to be provided by FDCs, including presentation of retained, intact antigen (Wykes M 1998; Bergtold A 2005; Qi H 2006).

The recent development of intravital multiphoton microscopy has allowed visualization of immune cells (e.g. antigen specific GC B cells, naïve resting B cells, and FDCs) when

they move through living lymphoid tissues. These powerful techniques (combining genetic and physical labelling techniques) can provide valuable insight into some of the GC development processes largely derived from histology. Three independent studies reveal consistent findings that alter the conventional picture of GC B cell behaviour (Schwickert TA 2007; Allen CD 2007a; Hauser AE 2007b). Although there are some debates, their observations all suggest that at least in mice, the phenotypes of GC B cells in these LZ and DZ may not be as different as what was previously thought (Fig 1.4). The studies by Allen and Hauser show that GC B cell proliferation occurs in both LZ and DZ. Allen (2007a) proposes that B cells may compete not only for antigen but also for T cell help. Migration of B cells between the LZ and DZ can occur at relatively low frequencies, but can also be quite extensive (Schwickert TA 2007). Therefore, Hause *et al* (2007b) revised the classic GC migration "cyclic re-entry model", and suggested an "intra-zonal circulation model" (Figure 1.4) (Detailed review regarding *in vivo* imaging of GC development see (Allen CD 2007b; Hauser AE 2007b). Currently germinal centre B cells can be observed by intra-vital microscopy only for very short time-spans, these observations are still controversial, and further studies are needed (Allen CD 2007b). Reanalysis of the intravital data using computer modelling further suggests that current observation times are too short to provide significant data on recirculation, and the data currently available are still compatible with the traditional 'cyclic re-entry model' (Figge MT 2008; Meyer-Hermann ME 2009).

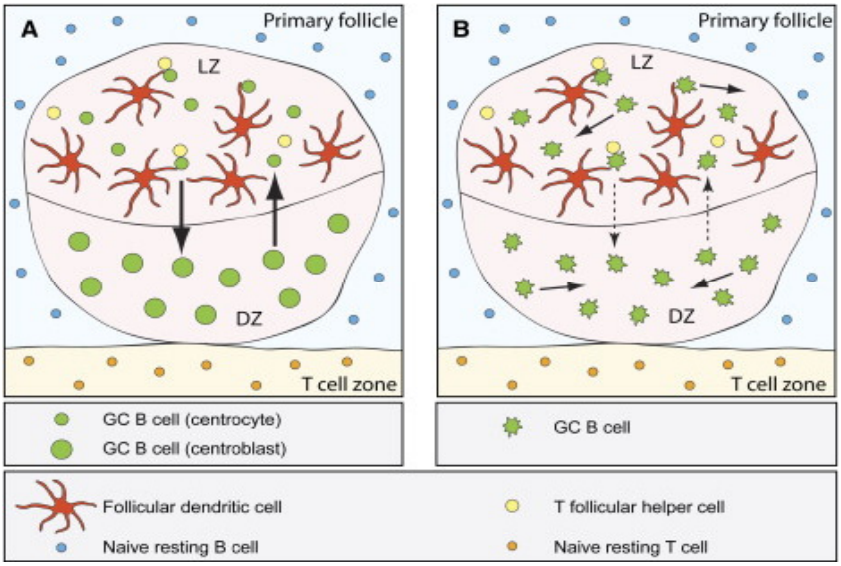

Figure 1.4: Conventional and revised views of germinal centre B cell morphology and migration

Germinal centers (GCs) form within the primary B cell follicle and consist of light zone (LZ) and dark zone (DZ), which are distal and proximal to the T cell zone, respectively. Follicular dendritic cells (FDCs) and T follicular helper cells (T_{FH}) localize within LZ.
A: The conventional view of the GC is that LZ B cells (centrocytes) are nonproliferating and relatively small, whereas DZ B cells (centroblasts) are large, dividing cells. According to the cyclic-re-entry model, there is a frequent passage of B cells between the LZ and DZ. **B**: The results of Hauser et al (2007b), Allen (2007a) and Schwickert (2007) indicate a modified picture of the GC in which B cells migrate extensively inside either the LZ or DZ, and can divide within either zone. Migration of B cells between the LZ and DZ can occur at relatively low frequencies, but can also be quite extensive (Taken from Bring 2007).

1.6 Plasmacytic differentiation

Exposure of B cells to TI antigens activates proliferation and formation of the extrafollicular foci of antibody-forming cells (AFC) (MacLennan IC 2003). These short-lived plasma cells secrete antibodies with relatively low affinity for antigen, usually of the IgM class and have no IgV region gene hypermutation, though a small amount of mutation may occur (Toellner KM 2002). Following exposure to TD antigens, one pathway of B cell differentiation is rapid clonal expansion of activated B cells, which

form the short-lived AFCs in splenic extrafollicular foci and lymph node medulla. This is homologous to the main B cell differentiation in the TI responses, providing rapid antibody production followed by fast antigen clearance (Manz RA 2002).

Several transcription factors have been found to be crucial for plasma cell commitment and differentiation. Pax5, a transcriptional repressor is essential for maintaining the phenotype of mature B cells, including naive, GC and memory B cells (Cobaleda C 2007). It has been shown that downregulation of Pax5 in mature B cells results in plasma cell differentiation (Delogu A 2006; Nera KP 2006). Inhibition of Pax5 may represent the initial step in plasma cell differentiation, resulting in the establishment of a 'pre-plasmablast' that secretes low amounts of antibodies. The pre-plasmablast is characterised by expression of Flt3 and Embigin genes which are otherwise repressed by Pax5 (Kallies A 2007b). This distinct stage is then followed by the upregulation of several genes that promote development into mature plasma cells.

The second crucial event is the downregulation of Bcl-6 (Falini B 2000; Basso K 2004), which allows GC B cells to differentiate into memory B cells or plasma cells. Bcl-6 stops the repression of Blimp1, which facilitates plasma cell differentiation (Shaffer AL 2000; Vasanwala FH 2002; Tunyaplin C 2004; Parekh S 2007). Combined BCR- and CD40-triggering is known to inhibit Bcl-6 expression in centrocytes.

Blimp-1 in the present study is used as a marker for terminal differentiation of B blasts into plasma cells. Blimp-1 knock-out studies show that GC detection and extrafollicular response occurs, but no plasma cells are produced (Shapiro-Shelef M 2003). Blimp-1 acts upstream of X-box binding protein 1 (XBP-1), a transcription factor that is required for the secretory phenotype of plasma cells (Reimold AM 2001; Iwakoshi 2003; Shaffer AL. 2004). Blimp-1 is capable of repressing the expression of genes that maintain B cell

identity, gene, such as Pax5 and CTIIA, and others that promote GC B cell proliferation such as Bcl-6 and c-myc (Shapiro-Shelef M 2005).

The transcription factor Interferon-Regulatory Factor 4 (IRF4) has been suggested to act upstream of (Sciammas R 2006), or in parallel to (Klein U 2006), Blimp-1 in the generation of plasma cells and isotype switching . IRF4 could inhibit Bcl-6 and induce Prdm1 which encodes Blimp-1 (Sciammas R 2006). Immunofluorescence studies have shown reciprocal expression patterns of IRF4 and Bcl-6 in GC B cells. The B cell subset expressing IRF4 is Bcl-6-negative, and the subset that expresses Blimp-1 is IRF4-positive (Cattoretti G 2006a). Recent work suggests that the expression of Blimp1, IRF4 and XBP-1 are independently regulated, but all three are required for the development of terminally differentiated plasma cells and/or initiation of plasma-cell differentiation (Reimold AM 2001; Klein U 2006; Sciammas R 2006; Kallies A 2007a). IRF4 is expressed in immature B cells in the bone marrow (Lu TT 2002) and has a biphasic pattern of expression in B cells. It is strongly induced within hours of B cell activation via T-independent stimuli. IRF4 protein expression can be seen during the transit of activated B blasts through the T zone. Although IRF4 is seen as a plasma cell differentiation related gene, rapid IRF4 expression is found in plasmablast as well as germinal centre B cell precursors. IRF4 expression is lost when B blasts move to follicles and differentiate into proliferating centroblasts (Marshall, Zhang *et al* submitted for publication, Caoretti 2006a).

Another transcription factor STAT3 may affect plasma cells differentiation (Fornek JL 2006). IL21 can induce Blimp-1 expression via activation of STAT3 (Ozaki K 2004). IL21 and STAT3 promote B cell differentiation *in vitro* (Ettinger R 2005; Good KL 2006), and are required for plasma cell development *in vivo* (Ozaki K 2002).

1.7 Memory B cell development

Memory B cells provide a rapid strong response, and produce antibodies of a greater affinity against recurrent antigens in comparison to those produced by the naive B cells (Toellner KM 1996; Anderson SM 2007; Tangye SG 2009). The formation and maintenance of memory lymphocytes are critically important for the design of vaccines and the treatment of immunodeficiency diseases, but the current knowledge of signals leading to memory B cell differentiation is less well defined than for plasma cell differentiation (Tarlinton 2006). Studies have defined that memory B cells (i.e. in serological memory) develop from GCs, and frequently, but not always, express switched antibody isotypes (Coico RF 1983). In mice, after TD antigen immunisation, memory B cells express class-switched antibody, CD38 and B220, present somatically mutant Ig V genes, and in addition, constitutively express co-stimulatory molecules CD80 and CD86 (Tarlinton 2006; Anderson SM 2007). In mice $CD80^+$ memory B cells are found in the blood, MZ and the follicle (Anderson SM 2007), and the spleen has been found to be the predominant site for the localisation of the long-lived memory B cells (Tangye SG 2009). Recent studies in mice have described that B-1b cells can form long-lasting memory cells in response to TI-2 antigen, and these memory cells express large amount of IgM, CD19 and CD11b, but little B220 and IgD (Alugupalli KR 2004). In humans the phenotype of memory B cells is less controversial than in mice (Liu YJ 1988; Dunn-Walters DK 1995). CD27, a marker associated with switched and mutated cells (Maurer D 1992; Klein U 1998), is found on B cells in the blood (Agematsu 1997). Some memory B cell subsets have been identified, such as IgM^+/IgM^-CD27^+, and $CD27^+/CD27^-IgG^+$. $CD27^-$ memory B cells express the inhibitory receptor Fc-receptor like 4 (FCRL4) (Ehrhardt GR 2005; Tangye SG 2006). IgM^+CD27^+ B cells were found in a patient, lacking GC, although there were fewer of these cells compared with the normal controls. These studies demonstrated that IgM^+CD27^+ memory B cells formation is dependent on GC.

Phosphorylated STAT5 may mediate human memory cell differentiation as opposed to plasma cell differentiation by controlling expression of Bcl-6 (Scheeren FA 2005). A role for Bcl-6 has also been suggested in memory B-cell development (Reljic R 2000). However, Bcl-6 deficient mice develop cells with a memory B cell-like phenotype (Toyama 2002), and *in vitro* experiments have shown Bcl-6 to inhibit the human memory B cell formation (Kuo TC 2007). The role of STAT5 in memory B-cell differentiation remains to be clarified. BAFF may have some roles for the survival of memory B cells, since memory B cells express BAFF-R and TACI, two receptors for BAFF (Tangye SG 2006). However, studies in mice demonstrated no effect on the memory B cells compartments after inhibiting BAFF signalling (Benson MJ 2008). Even though IRF4 is not required for memory cell formation, but it is essential for both induction of AID and hence CSR, and the production of plasma cells from naïve and memory B cells (Klein U 2006).

CD40 signalling may have an important role for memory B cell differentiation (Arpin C 1995). *In vitro* experiments show that human GC B cells cultured on CD40 ligand (CD154) expressing fibroblasts with IL-2 and IL-10 were found to generate a memory B cell phenotype, while the removal of CD154 from the system resulted in differentiation of the cells into plasmablasts (Arpin C 1995). It remains to be determined whether continued stimulation through CD40 directs the centrocytes towards the memory B cell pathway, whereas the release of CD40 signalling is a prerequisite for their differentiation into plasma cells *in vivo*. In addition, BCR signalling may regulate the activity of centrocytes. Some studies have suggested that low affinity B cells form GCs, and then become memory B cells (Smith KG 1997; Dal Porto JM 2002; Shih TA 2002a; Paus D 2006).

Chapter 2 Materials and Methods

2.1 Animals

Female and male mice of different strains and origins (Table 2.1) at the age of 6-16 weeks were maintained in specific-pathogen-free units at the Biomedical Services Unit (BMSU), University of Birmingham. All procedures were approved by the University Ethics Committee. The F_1 heterozygotes from QM x C57BL/6, and Cg1-Cre x ROSA26-eYFP were identified using PCR-typing (Section 2.10).

2.2.1 QM mice

Quasi monoclonal mice (QM) have a rearrangement in their V(D)J segments at the Ig heavy chain locus, and J_H sequence is replaced by the V_HDJ_H 17.2.25 segment. This introduced segment was taken from an affinity mature NP-specific BALB/c hybridoma. The mouse also has a targeted deletion of the κ light chain allele. When paired with any λ light chain, 17.2.25 is specific for the hapten NP (Cascalho M 1996). This mouse was designed to be 'monoclonal', however, only 60% of B cells respond to NP. Some are combining with a λ light chain that is not specific for NP. Furthermore, there are secondary rearrangements from recombination signal sequence that is present in the rearranged 17.2.25. Hence this mouse is called 'quasi monoclonal'.

QM×C57BL/6 F1 hybrid mice have one copy of rearrangement VDJ heavy chain genes and normal κ light chains. These mice have just around 5% of B cells that are NP-specific, because most B cells express κ genes (Marshall JL 2009, Thesis UoB). The response to NP-Ficoll in QM×B6 mice induces GCs and plasmablasts with similar kinetics to immunization with TD antigens if immediate T cell help is available (Vinuesa CG 2000).

Table 2.1: Animal used

Mouse strain	Source	Reference
C57BL/6	Harlan, U.K	
BABL/c	Harlan, U.K	
$QM^{NP/NP}$ x κ-/-	Prof. Matthias Wabl	Cascalho M (Science 1996)
$QM^{NP/—}$x C57BL/6 F_1 ($B6^{NP/WT}$)	Bred in BMSU, University of Birmingham, U.K.	Vinuessa CG (JEM 2000)
B1-8 x κ-/-	Prof. Michael Reth MPI of Immunology	Shih TA Y (Nat Immunol 2002)
$B1\text{-}8^{high}$ x κ-/-	Prof. Michael Reth MPI of Immunology	Shih TA Y (Nat Immunol 2002)
ROSA26eYFP	Jackson Lab	Soriano P. (Nature Genetics 1999)
Cγ1-Cre	Dr. Stefano Casola (IFOM, Milan)	Casola S (PNAS 2006)
Cγ1-Crex ROSA26eYFP	Bred in BMSU University of Birmingham	
IL-4 deficient (BALB/c background)	Charles River Laboratories France	Kuhn R (Science 1991)
IL-4Rα deficient (BALB/c background)	Prof. James Alexander (University of Strathclyde)	Mohrs M (J. Immunol. 1999)
IL-6 deficient	Charles River Laboratories France	Kopf M (Nature 1994)
IL-10 deficient	Charles River Laboratories France	Kuhn R (Cell 1993)
IL-13 deficient (BALB/c background)	Charles River Laboratories France	McKenzie GJ (Immunity 1998a)
IL-22 deficient (BALB/c background)	Prof. Chris Bukley (University of Birmingham)	Zheng Y (Nat Immunol 2008)
CD28 deficient (BALB/c background)	Prof. Granham Anderson (University of Birmingham)	Shahinian A (Science 1993)
CD30 deficient	Prof. Peter Lane (University of Birmingham)	Piggig SD (J. Immunol 1999)
OX40 deficient	Prof Peter Lane (University of Birmingham)	Amakawa R (Cell1996)
CD30OX40 double deficient	Bred in BMSU University of Birmingham	Gaspal F (JEM, 2005)

2.2.2 B1-8 and B1-8 ^{high} mice

The B1-8 mouse is also a powerful model to investigate the TI-2 (NP-Ficoll) immune response. It is designed on C57BL/6 mouse background, and contains V_H 186.2-DFL16.1-Jh2 derived from NP-binding antibody B1-8 (Bothwell AL 1981; Reth M 1986; Zou YR 1993), and λ1 only light chain by mice homozygous for the (Cκ) mutation (Zou YR 1993). This kind of light chain can provide an efficient way to quantitate V_HB1-8 expression in the B cell population (Sonoda E 1997). Mutation of Trp→ Leu at codon 33 of B1-8 increases the affinity for NP by a factor of 10 (B1-8 ^{high}), whereas the four amino acid changes found in hybridoma 3C52 decreases NP-binding by a factor of four (B1-8 ^{low}) (Allen D 1986). For both B1-8^{high} and B1-8^{low} genes, an additional silent TGT→ TGC mutation was introduced at codon 96 to avoid V_H replacement (Reth M 1986). These antibody genes are expressed in the endogenous locus and are therefore subject to physiologic regulation, including switch recombination. Furthermore, these gene recombinations do not affect the populations of pro-, pre-, immature, transitional, follicular and MZ B cells in bone marrow and spleen (Shih TA 2002b).

2.2.3 Cγ1-Cre × ROSA eYFP mouse

Cγ1-Cre mouse

Cre/*lox*P-mediated conditional gene targeting is an approach to tackle proliferation, differentiation and selection of GC B cells *in vivo*. Cγ1 (IgG1 constant region locus)-Cre mice were developed on the C57BL/6 genetic background. An internal ribosome entry site (IRES) followed by the Cre-coding sequence in 129-derived ES cells was inserted into the 3' region of the Cγ1 locus between the last membrane-coding exon and its polyadenylation sits (Fig 2.1A). This approach allows for the expression from the Cγ1 locus of a bicistronic mRNA consisting of the Cγ1 and the Cre transcript, respectively (Casola S 2006) (Casola S 2004). This mouse strain promotes conditional gene targeting

in the majority of GC B cells generated, IgG1 memory cells and plasma cells in response to immunization with TD antigens. Driving Cre expression from the Cγ1 locus allows to track the fate of B cells undergoing sterile Cγ1 transcription and thus to investigate to what extent this process controls the targeting of CSR *in vivo* (Casola S 2006).

Rosa26-eYFP reporter stain

This transgenic mouse model based on C57BL/6 background, is designed mainly by using Cre-*lox*P system as well. Firstly a Cre-dependent *Lac*Z reporter strains is produced by targeted insertion of a *Lac*Z gene, proceeded by a *lox*P- flanked (floxed) strong transcriptional termination sequence (tpA), into the ubiquitously expressed ROSA26 locus (Soriano 1999). When the mice are crossed with Cre-expressing transgenic mice, the Cre-mediated excision of the floxed termination sequence leads to constitutive *lac*Z expression. In these doubly transgenic animals, a *lac*Z gene is turned on in cells expressing Cre recombinase, as well as daughter cells. These mice are useful for cell lineage tracing experiments as well as for monitoring the expression of Cre transgenes, but *lac*Z expression cannot easily be detected in living tissue. EYFP (enhanced yellow fluorescent protein) as a report offers an advantage for monitoring the expression of Cre and tracing the lineage of these cells and their descendants in living tissue (Soriano 1999; Mao X 2001; Srinivas S 2001). EYFP as report was inserted into the ROSA26 locus, preceded by a *lox*P-flanked stop sequence, in a genetic targeting vector (Fig 2.1B), and then this targeting vector was then electroporated into the ES cell. The mouse strain was tested by crossing with Cre-expressing transgenic mice, and the Cre-mediated excision of the floxed termination sequence leads to constitutive eYFP expression. If mice have a silencer surrounded by two *lox*P sites and the colour marker, eYFP is only expressed in the cells that express Cre. Casola (2006) studied the efficiency of Cre-recombination *in vivo* at the single cell level, Cγ1-Cre mice were crossed with a ROSA26eYFP reporter strain (Cγ1-Cre×ROSA eYFP), and finally showed that eYFP expressed essentially in all

B cells after T help type 2 like activation (Fig 2.1).

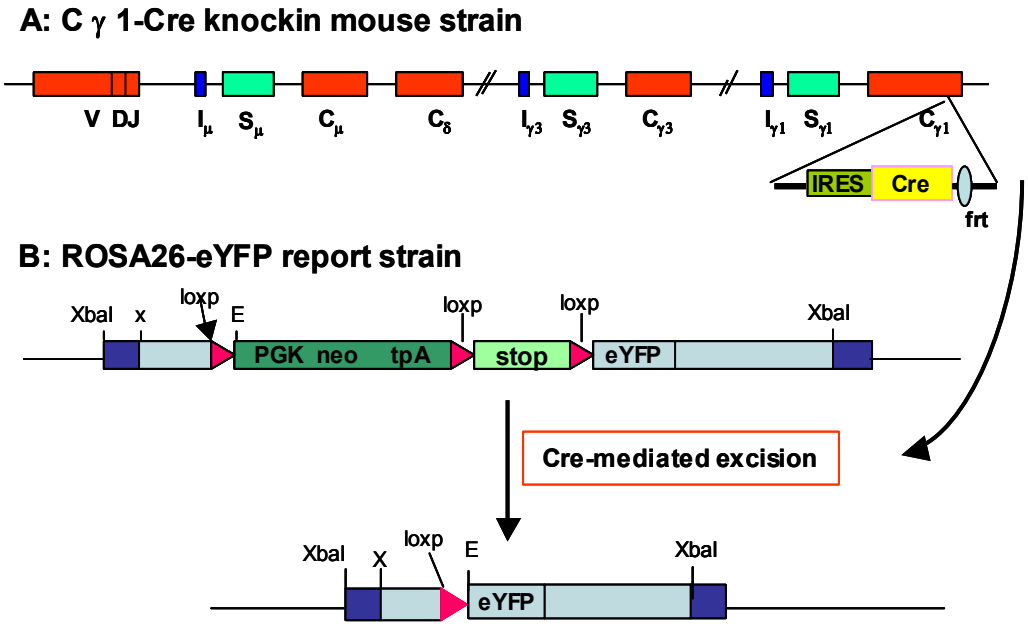

Figure 2.1: Gene map of Cγ1-Cre×ROSA26-eYFP mice
A: the basic gene map of Cγ1-Cre knockin mouse strain. **B**: gene map of ROSA26-eYFP report strain. When Cγ1-Cre mice are bred to the R26-eYFP, Cγ1 transcription expression will induce Cre, Cre recombinase has to induce excision of *lox*P flanked reporter expression, and finally reporter expression will be detected.

2.2 Antigens and Immunizations

For immunization, (4-hydroxy-3-nitrophenyl)-acetyl-CGG (NP-CGG) was used at 250μg/ml suspended in sterile phosphate buffered saline (PBS) (Sigma UK) and injected intraperitonally (*i.p.*). NP-CGG, a conjugated protein, was prepared using NP-OSu (Cambridge Research Biochemicals, UK) dissolved in dimethylformamide (DMF) (Sigma, Poole, UK) at 200 mg/ml. CGG (Sigma) was dissolved in PBS at 5 mg/ml. 200 μl of NP was added to 1 ml of the protein solution and incubated for 2 hours at room temperature on a rotating mixer, then extensively dialysed against PBS pH 7.4. The final conjugation ration is NP_{18}-CGG, a kind gift from Ms Chandra Raykundalia. NP-CGG/CGG was precipitated in 9% aluminium potassium sulphate (in H_2O, sterile) for primary immunization (Nossal GJ 1990). The alum precipitate was made with 1 part

NP-CGG/CGG (5mg/ml) to 1 part Alum. This is made up to 500 µl with sterile PBS and the pH adjusted to 6.5. This was kept in the dark in room temperature for 1 hour, and was then washed twice in PBS. The pellet was resuspended in 50µg /200µl PBS per mouse and if required 10^7 *Bordetella pertussis* (LEE laboratories, BD, USA) was added per mouse. For footpad injection, 20µg/20µl NP-CGG +5×10^8 *B.p.* is given per pad.

Sterile NP-Ficoll (Biosearch Technologies, CA) was diluted in sterile PBS during studies on B cell response in QM×B6 mice or B1-8/B1-8^{high} mice, and usually 30µg/200µl of NP-Ficoll was given to each mouse via *i.p.*. NP-Ficoll immune complex preparations were based on a ratio of hapten /antibody antigen-binding site of 1:1. One part of NP_{28}-Ficoll (6µg) and 5 part of anti-IgD or anti-IgM (30µg) were mixed in 200µl of PBS just before *i.v.*injection.

Some mice were injected intravenously (*i.v.*) 2×10^8/200µl sheep red blood cells (SRBC, TCS Biosciences). SRBC were prepared by washing three times, followed by centrifugation and resuspending in PBS.

2.3 Anti-NP antibody production

2.3.1 Anti-NP antibody production

We developed a number of hybridoma cell lines to produce large amounts of anti-NP antibodies with different affinity/avidity and isotypes in a large amount. This project was in collaboration with Dr Margaret Goodall. B cells were removed from the spleen of B1-8/B1-8^{high} (IgH^a allotype) mouse at day 4 after immunized with 30µg/200µl of NP-Ficoll via *i.p.*. Using polyethylene glycol B cells are then fused with myeloma tumor cells, which can grow rapidly and indefinitely in culture, and produce large amount of antibodies. Viable hybridomas are selected and screened for NP-specific antibodies by

ELISA. The single antibody secreting hybridomas with the highest absorbance are selected and grown. Other hybridomas are frozen down at a concentration of 1.0×10^7 cells/ml for long term storage and one is selected for cloning by limiting dilution to generate a stable and generation cell line. Wells with growing cells following cloning antibody secretion are screened by ELISA again. Isotyping of the positive hybridomas will be included. Supernatant from a specific hybridoma will be grown up to approximately 500 ml. Antibody was affinity purified from supernatant according to specific isotype, and then extensively dialysed against PBS pH 7.4. The antibody purity was simply checked by running on SDS-PAGC gel (PIERCE). Some antibody (i.e. IgM) with low purity was further purified by affinity chromatography using specific monoclonal antibody. On rate, off rate, and avidity of all antibodies for binding antigen (NP) have been checked by BioCore (BiaCore 3000 GE), and the data was analyzed by the related software. All hybridoma have been frozen down for long term storage after get amount of antibodies. Hybridomas we produced at moment and imported from Cologne (Klaus Rajewsky lab) are listed below.

Table 2.2: Anti-NP hybridoma list

	Name	**Isotype**	**Affinity**	**Specificity**	**Allotype origin**
1	Clone 82	IgM^a	2.5×10^{-6}	Anti-NP	QM (IgH^a)
2	NP2.315	IgM^a	2×10^{-6}	Anti-NP	B1-8 (IgH^a)
3	NP2.248	IgM^a	2×10^{-6}	Anti-NP	B1-8 (IgH^a)
4	NP1.197	IgM^a	2×10^{-7}	Anti-NP	$B1\text{-}8^{hi}$ (IgH^a)
5	NP1.298	IgM^a	2×10^{-7}	Anti-NP	$B1\text{-}8^{hi}$ (IgH^a)
6	231	$IgG3^a$	2×10^{-7}	Anti-NP	$B1\text{-}8^{hi}$ (IgH^a)
7	240	$IgG2a+b^a$	2×10^{-7}	Anti-NP	$B1\text{-}8^{hi}$ (IgH^a)
8	18-01-16	$IgG1^a$		Anti-NP	BALB/c (IgH^a)
9	B1-8 IgD	IgD^b	2×10^{-6}	Anti-NP	C57BL/6 (IgH^b)
10	B1-8 IgM	IgM^b	2×10^{-6}	Anti-NP	C57BL/6 (IgH^b)
11	S8	$IgG2a^b$	5.8×10^{-9}	Anti-NP	C57BL/6 (IgH^b)
12	S43-10	$IgG2a^b$	2.4×10^{-8}	Anti-NP	C57BL/6 (IgH^b)

2.3.2 BiaCore for checking antibody avidity

For analysis of antibody affinity, we employed the BiaCore system, which utilizes surface plasmon resonanace (SPR) as a means of detection, to measure the real-time interaction between NP protein conjugates and anti-NP antibodies. NP_{15}-BSA was covalently attached to the sensor chip via the amine coupling method. Carboxyl groups on the surface of the sensor chip are activated with a mixture of EDC and NHS (BiaCore location) to give reactive succinimide esters. The ligand is then passed over the surface and the esters react with primary amine groups and other nucleophilic groups to link the ligand covalently to the dextran matrix. Ethanolamine is then passed over the chip to deactivate excess reactive groups (the detail methods according to manufacture's instructions). Ligand was prepared in acetate buffer (pH 4) and run at a flow rate of 5µl/min for 30 min at 25°C. Antibodies were diluted to various concentrations (from 15.625µM to 2mM) in degassed PBS. Each antibody dilution was run for a period of 1800 seconds at a flow rate of 5µl/second. All experiments were performed at 38°C. Data was analysed on the BIAevaluation software (BIAevaluation 3.2RC1) and the background was measured by passing the analyte through a flow cell containing BSA immobilised to the chip. This background response was then subtracted from the sensorgram before analysis.

Table 2.3: Reagents required for BIAcore surface plasmon resonance

Solution	
EDC	0.4M 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide in water
NHS	0.1M N-hydroxysuccinimide in water
Ethanolamine	1M ethanolamine-HCL pH 8.5

2.4 Tissue section for experiment

2.4.1 Tissue section preparation

Spleens were removed from sacrificed mice, and after carefully taking away excess fat,

they were immediately snap frozen in liquid nitrogen (N_2). The tissues were stored in a -80°C freezer until use. When ready for use, the tissues were mounted in pre-cooled O.C.T. Compound and quickly freeze-sprayed. 5μm sections were cut using a cryostat (Bright Instruments, Huntingdon, UK), and picked onto multispot glass slides. The sections were air-dried under fan at room temperature for 1 hour, and then the air-dried sections were fixed in cold acetone (4°C) for further 20 min. Fixed slides were again air-dried for 10 min before finally being stored at -20°C for later use.

2.4.2 Immunohistochemistry staining

Frozen sections were warmed to room temperature under fan for 10 min, and then rehydrated by immersing in Tris buffer (pH 7.6). After rehydration the sections should not be dried-out and were made sure covered by solution. Rehydrated sections were covered with appropriately diluted primary antibodies (concentrations and details shown in Table 2.4), and incubated for 1 hour at room temperature. Secondary antibodies were absorbed 30 minutes before use in 10% normal mouse serum to eliminate anti-mouse cross-reactivity. At the end of the first incubation slides were washed for 5 minutes. Secondary antibodies (concentrations and details shown in Table 2.5) were added to corresponding sections, and left to react with sections for 45 minutes. Excess antibodies were washed away in Tris buffer (pH 7.6), when staining with biotinylated secondary antibodies. Tertiary stains were used when necessary. Streptavidin complex (Vector, UK), prepared 30 minutes before use, was added to sections and left to react for 30 minutes. After washing, stains were developed using first the peroxidase substrate solution for horseradish peroxidase (PX) conjugated to secondary antibodies, and then alkaline phosphate substrate solution for biotinylated antibodies (with five minutes washing between adding two substrate solutions, and substrate solution have to make fresh before using). Developed slides were washed twice in dH_2O and left to dry (minimum 30minutes) before being mounted in immunomount (Thermo, Basingstoke, UK).

Table 2.4: Primary antibodies

Target	Isotype	Dilution	Clone	Manufacturer
CD3	Rat anti Ms	1/2500	17A2	Got from P. Lane lab
IgD	Shp anti Ms	1/1500	Polyclonal	abcam
IgD	Rat anti Ms	1/1000	11-26C.2a	BD PharMingen
IgG1	Rat anti Ms	1/300	LO-MG1-2	Serotec
IgG	Rat anti Ms	1/200	COCKTAIL	Serotec
IgG3	Rat anti Ms	1/200	LO-MG1-13	Serotec
IgM	Rat anti Ms	1/500	LO-MM-9	Serotec
Caspase3	Rb anti act. Casp.3	1/100	C92-605	BD PharMingen
NP	NP conjugated Shp	1/3000		Got from P.Lane Lab
CXCR4	Rat anti Ms	1/100	2B11	BD PharMingen
IRF4	Goat anti Ms	1/400	M-17	Santa Cruz
CD138	Rat anti-Ms	1/200	281-2	BD PharMingen
PNA biotin		1/200		Vector
IgM^a FITC	C57BL/6	1/200	DS-1	BD PharMingen
IgM^b Biotin	BALB/c	1/100	AF6-78	BD PharMingen
Bcl-6	Rb anti-Ms	1/30	N-3	Santa Cruz

Table 2.5: Secondary and Tertiary antibodies

Target	Dilution	Manufacturer
Rb anti Rt biotin	1/600	Dako
Rb anti Rt PX	1/50	Dako
Dnky anti Shp biotin	1/100	The Binding Site
Dnky anti Shp PX	1/100	The Binding Site
Swine anti Rb biotin	1/400	The Binding Site
Rb anti FITC	1/300	Dako
Swine anti Rb Immunogloblins	1/100	Dako
Rabbit PAP	1/100	Dako

Table 2.6: the receipts of buffer and substrate solution

Tris buffer pH7.6	1.5L Physiological NaCl (7.5G/L) 1.5L 0.1 M Hcl 1.0L 0.2M Tris
Tris buffer pH9.2	As above but pH made up to 9.2
Peroxidase substrate solution	Dissolve 1 tablet (10mg) of 3,3'- Diaminobensidine tetrahydrochloride (DAB) (Sigma) in 15 ml Tris buffer (PH7.6), and the solution is filtered to 10 ml. One drop of 30% hydrogen peroxide (Sigma) was added to the solution to activate the enzyme

Alkaline phosphate substrate solution	Dissolve 8mg of levanisole (Sigma) in 10 ml of Tris buffer (pH 9.2). 4mg of Naphthol AS-MS phosphate (Sigma) was dissolved in 380µl of dimethyl formamide in a glass bijox, and this solution was added into the middle of the levanisole solution. Finally add 10 mg of Fast Blue salt (Sigma) to that mix, filter.

2.4.3. Immunohistology for confocal microscope

Normal frozen tissue section was cut at 5µm, and picked on normal multispot glass. EYFP spleen tissue was fixed in 4% formaline + 10% sucrose in PBS for 4 hours at 4˚C, and then snapped frozen in liquid N_2 (Kusser KL 2003). This tissue was cut at 5µm, and picked on Super Frost@ plus glass slide. Antibodies were incubated at room temperature in a dark shaking chamber. Sections were washed by PBS for 5 min, and were firstly adsorbed with PBS supplemented with 5% horse serum for 10 mins. Primary antibodies were incubated for 1 hour at room temperature in PBS supplemented with 10% BSA, then washed for 5 mins in PBS. Subsequent antibodies were incubated for 30 mins. Sections were immersed in Hoechst 33258 (Sigma) for 1 mins for nuclear staining, then mounted using Prolong@ gold antifade reagent (Invitrogen), cure for 24 hours in dark at room temperature and finally kept in the dark at -20˚C. A Zeiss confocal LSM 510 microscope (Zeiss, Germany) was used to visualise staining with images captured and processed using Zeiss LSM Image Examiner software (Zeiss).

Table 2.7: Primary Antibody not listed in the normal histological staining

Target	Isotype	Dilution	Clone	Manufacturer
CD3	Hs anti Ms	1/200	145-2C11	BD PharMingen
IgD alexa@ 647	Rt anti Ms	1/100	11-26c	BD PharMingen
IgG1 alexa@ 633	Gt anti Ms	1/400	Polyclonal	Mol. Prob. Invitrogen
GL-7	Rt anti Ms	1/100	GL-7	BD PharMingen
Fas	Hs anti Ms	1/100	Jo2	BD PharMingen
CXCR5	Rt ant Ms	1/100	2G8	BD PharMingen
Ki67	Rb anti Ms	1/400		J. Gerdes, Germany
BP3	Rt ant Ms	1/500	KT157	Cedarlane Lab
B220 FITC	Rt anti Ms	1/100	RA3-6B2	BD PharMingen

Table 2.8: Secondary Antibody

Target	Dilution	Manufacturer
Gt anti-FITC alexa@ 488	1/100	Molecular Prob Invitrogen
Gt anti Hs Cy5	1/100	Jackson ImmuoResearch
Dnky anti Rt Cy3 /Cy5	1/300	Jackson ImmuoResearch
Dnky anti Shp Cy3/Cy5	1/300	Jackson ImmuoResearch
Gt anti-Rb Cy3/Cy5	1/300	Jackson ImmuoResearch
Str Alexa @ 405	1/50	Molecular Prob Invitrogen
Str.Cy3	1/400	Jackson ImmuoResearch
Rb anti Gt biotin	1/200	Dako

2.4.4 Flow cytometry staining

Cells were prepared from freshly collected spleens. The cells in RPMI-1640 (Sigma), supplemented with 10% fetal calf serum (GIBCO) and 1% penicillin/streptomycin (Invitrogen), were spun down again and resuspended in FACS buffer (1% fetal calf serum and 5mM EDTA in sterile PBS). All procedures were carried out at 4°C or on ice to eliminate cell death and stop the cell cycle. Approximately 1×10^6 cells were used for each staining, and they were kept in 100µl FACS buffer. The appropriate type and amount of antibodies (Table 2.9) were added to each cell sample, and incubated for 20-30 minutes. At the end of this incubation the cells were washed two times with FACS buffer by pelleting at 1,200 rpm for 4 min. When biotinylated antibodies were used, a second incubation was carried out. Cells were incubated for 20 min and washed as previously described. IRF4 was detected after cells were permeabilizated and fixated with BD cytofix/cytoperm buffer (APC BrdU Flow Kit, BD Pharmingen) by first using Goat anti-mouse IRF4 (1/200 Santa Cruz), and followed by donkey anti-goat Cy5 (1/500 Jackson ImmuoResearch). Data acquisition was performed on a Becton Dickinson FACS Cyan, and analysed using FlowJo software (FlowJo, Ashland).

Table 2.9: Antibodies used in FACS

Reagents	Dilution	Clone	Source
CD16/32 purified	1/200	24g2	BD PharMingen
B220 Pacific Blue	1/50	RA3-6B2	BD PharMingen
NP PE	1/2000		Got from P.Lane Lab
CD4 PE	1/500	GK1.5	BD PharMingen
CD3 PE	1/50	145-2C11	BD PharMingen
Fas PE-Cy7	1/100	Jo2	BD Pharmingen
CD138 Biotin	1/100	281-2	BD Pharmingen
Str. Per CP-Cy5.5	1/200		BD PharMingen

2.5 Assessment GC size and Quantitation of antigen- (NP) specific B cells and plasma cells

We used slides that carry four stained serial sections. Germinal centres were identified as IgD^- areas surrounded by IgD^+ follicular mantle. To quantify the GC area, we counted the number of intercepts on a $100mm^2$ eyepiece graticule for total GC or NP specific GC at a magnification of ×25. The total spleen section was measured by counting intercepts on a $100mm^2$ acetate overlay at a magnification of ×4 (Weibel 1963). The concentration of some specific cells (e.g. NP^+) was expressed as the number of cells counted per $100mm^2$ acetate at a magnification of ×25.

To quantify the number of $IRF4^+$ cells at the GC-T zone interface, cells were counted in the area of 1× acetate wide at a magnification of ×25 (Fig 2.2), and the sum adjusted for the spleen size, the final data was presented by related to the spleen size or the whole GC size. The concentration of $IRF4^+$ cells in the follicle was measured as the number of cells counted in follicles on the whole spleen section, presented by related to the spleen size.

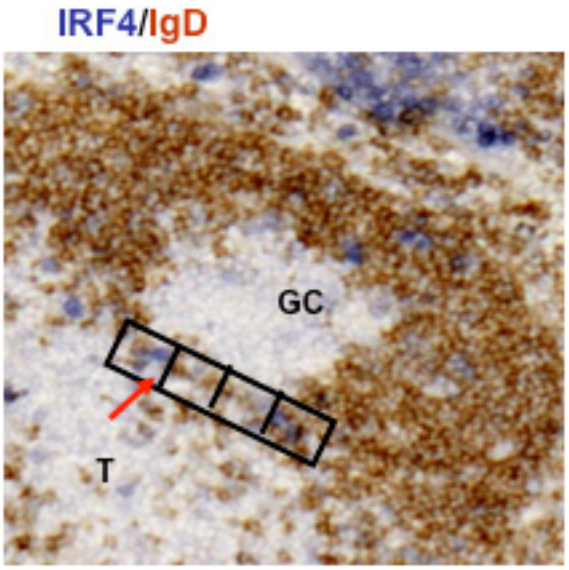

Figure 2.2: Quantification of IRF4^{+} at the GC-T zone interface
The IRF4+ cells in the GC-T zone interface of 1 × acetate wide under the a magnification of ×25 were counted. The GC-T zone interface size from the picture: 3 (long) × 1 (wide).

2.6 Technique of quantifying brown/blue staining of immune complex in GC

To measure the appearance and disappearance of IgM^{a} immune complex (IC) in GCs, we used the ImageJ software (Rasband 1997-2009) to quantify the DAB derived brown and the Fastblue derived blue immunohistological staining for IC on FDCs. IgM^{a} was usually stained brown, while IgM^{b} was stained in blue. At early stages of the response IgM^{a}-IC clearly presents in GC LZ (Fig 2.2 A). To separate brown from blue staining, ImageJ was used to do colour deconvolution using inbuilt vectors for FastBlue and DAB. This procedure converts the image into a dual channel image (Fig 2.2 B) (Ruifrok AC 2001) or the detail function instrument on the website). Regions of interest were drawn around areas representing IC on the FDC network (Fig 2.2 C). The mean pixel values in the channel representing brown and blue staining were measured (Fig 2.2D). From this, the ratio of brown/blue is calculated. One or two areas of interest per GC were chosen, and in total 10-20 regions of interest per individual tissue section.

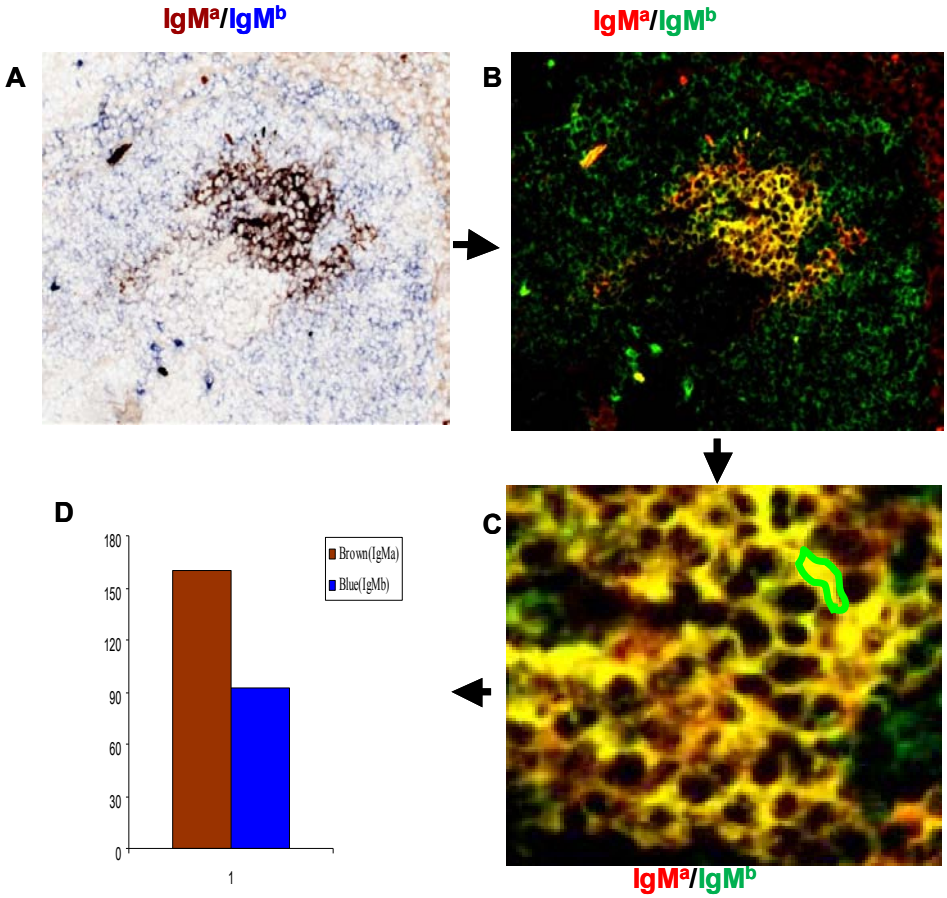

Figure 2.3: Technique of quantifying blue and brown staining

ImageJ was used to separate these colours using colour deconvolution, which converts brown and blue to a red/green dual channel image (**B**). Then regions of interest are drawn on the FDC network (**C**), and mean red and green pixel values are determined for this area (**D**). **A**: Original staining, **B**: Colour deconvolution, D: Colour intensity value of brown and blue

2.6.1 Technique of quantifying IRF4 blue staining on spleen section

ImageJ software was used to assess the IRF4 expression on tissue section at the time points after immunization. This is the similar technique to quantify brown/blue staining of immune complex in the GC. This procedure converts the image into a dual channel image. The areas of interest were selected by drawing at the same size in the different sites such as the marginal zone, follicle, outer T zone, middle T zone, GC, $IRF4^+$ plasma cell area, and IRF4 low/negative red pulp as negiative control. The mean pixel values in the channel representing blue staining were measured. 5 or 6 areas per site/white pulp were chosen, and in total 10-30 regions of interest per individual tissue section.

2. 7 Detection of serum antibody levels

2.7.1 Serum preparation

Blood was taken from the heart while the mice were under anaesthesia with CO_2. The blood samples were left in 37°C water bath for 60 minutes, and spun at 13,000 rpm for 5 minutes. The clear supernatants were collected and stored at -20°C.

2.7.2 ELISA

This technique was used to detect serum antibody level specific for antigen. 96 well flat-bottomed plates (Nunc) were coated overnight at 4°C with 100µl of some antigen in coating buffer (Table 2.10). For detection of high-affinity NP-specific antibodies (IgG), NP_2-bovine serum albumin (NP_2-BSA; 5µg/ml) was coated onto plates. As for total titre of NP-specific abs (IgG and IgM), NP_{15}-BSA (5µg/ml) was used. All NP conjugated proteins were prepared by Mrs C. Rayundalia. The plates were washed with wash buffer, and blocked for 1 hour at 37°C with 200µl blocking buffer per well. The blocking buffer

was tipped of and washed three times with wash buffer. Sera (1/30 or 1/100 dilution at start) were added into wells and serial tripling dilutions were made using dilution buffer. This incubation was for 1hour at 37°C. At the end of the incubation period, the serum was carefully washed away with wash buffer for three times. Anti-Ig antibodies were diluted into appropriate concentrations (Table 2.11) with dilution buffer, and 100µl of the diluted antibody was added into each well. After one hour of incubation the plates were washed for three times, and 100µl of substrate solution was added into each well. The development took approximately 20 min in a 37°C incubator or 2 hours for weak reaction such as high-affinity IgG1. The absorbance of the developed substrate solution was read at a wavelength of 405 nm. Standard calibration curves were plotted from the positive control sera and the test serum titres calculated as a relative value of these standard controls.

Table 2.10: Reagents used in ELISA

Reagents	**Recipe**
Coating buffer	Na_2CO_3 1.95g, $NaHCO_3$ 2.93g in 1L dH_2O, pH=9.6
Wash buffer	0.1M PBS (pH=6.8)+ 0.05% Tween (Sigma)
Blocking buffer	0.1M PBS + 1% BSA (Sigma)
Dilution buffer	0.1M PBS + 0.05% Tween + 1% BSA
Substrate solution	Dissolve substrate NpNN (N2770 Sigma)(one gold and one silver tablet) in 20ml dH_2O

Table 2.11: AP-conjugated antibodies used in ELISA

Ab Specificity	**Coating-Antigen**	**Dilution**	**Source**
Goat anti Ms IgM (AP)	* NP_2-BSA	1/2000	Southern Biotech
Goat anti Ms IgG (AP)	NP_{15}-BSA, NP_2-BSA	1/1000	Southern Biotech
Mouse anti Ms IgM^b biotin	# NP_{15}-BSA	1/500	BD PharMingen

*: Because IgM is multi (deka)-valent, the titre was just measured on NP_2-BSA coated plates.

#: Mouse anti-Ms IgM^b: monoclonal antibody, and one more step (Alkaline Phosphatase Streptavidin Vector) to detect IgM^b production by comparing to normal method. So the results were much weaker, only NP_{15}-BSA coating plate was used.

PS: Goat anti-Ms IgM/IgG AP antibodies are polyclonal. IgM^b clone: AF6-78

2.8 mRNA detection

2.8.1 Total mRNA extraction

5µm spleen section was taken and saved in -80°C until use. RNA was extracted by using the RNeasy Mini kit (Qiagen, UK), incorpotating the use of QIAshredder columns (Qiagen), according to the manufacturers instructions to ensure complete homogenization of the tissue. The resulting RNA was eluted in 30µl of RNAse free water and stored at -80°C until needed.

2.8.2 Production of cDNA

10µl of RNA solution was mixed with 1ul of random oligo-dH6 (0.5µg/ul) (Roche, Lewes,UK) and denatured at 70°C for 10 mins. This was shock cooled on ice and 9ul of reverse transcription mix was added. The following reagents were added to each sample:

10µl	RNA sample
0.5µl	dH_2O (Gibco-BRL)
4µl	5×first strand buffer (Invitrogen, Paisley, UK)
2µl	0.1M DDT
1µl	dNTP (10Mm)
0.5µl	RNAse Inhibitor (RNAguard, Pharmacia)
1µl	Moloney murine leukemia virus (M-MLV) reverse transcriptase (Gibco-BRL)

This was mixed and heated for 1h at 41°C, and then for 10 minutes at 90°C to inactivate RT. The cDNA was stored at -20°C.

2.8.3 Semiquantitative real time PCR

This is to quantify in cDNA the expression of the gene of interest such as switch transcripts, transcription factors, chemokines, or cytrokins. PCR was performed by adding 1µl of cDNA prep to each well of a 384 well plate (Applied Biosystems) followed by the required primers and probe mixtures (For sequences see Appendix table 3), and TaqMan Universal PCR master Mix (Applied Biosystems). The plate was then covered with clear adhesive foil (Applied Biosystems) and centrifuged to remove any air bubbles (1200rpm, a few seconds). Along with the amplification of the targeted gene, the internal

housekeeping gene β-actin or β_2-microglobulin was also amplified so that relative quantities of gene amplification could be assessed regardless of the number of cells harvested to produce each cDNA preparation. The probe for β-actin was VIC-labelled, and β_2-microglobulin is NED lablled (Applied Biosystems) so as not to interfere with the FAM-label. The plate was run in an ABI 7900 Real-Time PCR machine (Applied Biosystems) with a cycling programme as follows:

- 2 minutes at 50^{o}C
- 10 minutes at 95^{o}C

Then 40 cycles of

- 15 seconds at 95^{o}C
- 1minute 60^{o}C

Analysis of fluorescence signals was perfomred using SDS 2.2.2 software (Applied Biosystems). A threshold was set within the logarithmic phase of the PCR. The cycle number (Ct) at which the signals for target gene and house-keeping gene were recorded for each sample. The relative quantity of expressed target gene mRNA was calculated by subtracting the Ct for the housekeeping gene from that of the target gene (ΔCt) and then calculating $2^{-\Delta Ct}$.

2.8.4 PCR Arrays

TaqMan low-density real-time PCR arrays (Applied Biosystems) were designed in a 96-gene format. Genes chosen were reported to be differentially expressed during early plasmablast or germinal centre cell differentiation ((Klein U 2003; Tarte K 2003; Underhill GH 2003; Shapiro-Shelef M 2005). RNA was isolated as above and mixed with QuantiTect Probe PCR Kit (Qiagen). This was added to the TaqMan Low-Density Array, and PCR was performed in a 7900HT Real-Time PCR System (Applied Biosystems) according to manufacturer's recommendations. Array data was analyzed using TMEV (http://www.tm4.org) and Applied Biosystems SDS 2.2.2 software. Relative signal per cell was calculated as above with β2-microglobulin as the house keeping gene control.

2.9 Laser capture Microdissection for qRT-PCR

For laser capture microdissection (LCM), snap-frozen spleen sections were processed as described as for normal slide section (see 2.4.1), but serial sections were collected on PALM MembraneSlides NF (PALM) and stained for 3 min with 1% w/v cresyl violet (Sigma-Aldrich) in ethanol after hydration in 100%, 70%, and 50% ethanol. Slides were then washed again quickly in 50%, 70%, 100% ethanol sequentially and air-dried. Laser microcapture was performed using a Microbeam HT microscope (PALM Microlaser Technologies). However additional localisation techniques are needed to identify splenic compartments with confidence, and GCs themselves cannot be identified by cresyl violet staining alone. Two additional sections were thus cut before the sections to be used for microdissection, and two additional sections cut after. These four were stained by immunohistochemical techniques (IgD/IRF4). Each section was photographed in entirety. By cross reference between composite photomicrographs of the immunohistochemically stained slides and the cresyl-violet stained sections I identified GC (IgD^- area), follicle (IgD^+), central T zone, the GC-T zone interface (containing $IRF4^+$ cells), plasmablast/plasma cell rich extrafollicular foci ($IRF4^+$), and plasma cell sparse areas of red pulp ($IRF4^-IgD^-$) for microdissection. Finally membrane only was selected as a negative control (Fig 2.4).

Spleen tissue was selected from d5 after NP-CGG response in the carried-primed C57BL/6 mice, due to the maximum $IRF4^+$ cells appearance on the GC-T zone interface at that time point. The 10-20 similar microdissected areas were catapulted into RNeasy buffer (Qiagen) in an mRNA free eppendorf and RNA isolation processed by using the RNeasy Micro kit (Qiagen, Crawley, UK)(Erickson HS 2009; Mohr E 2009). RNA storage and cDNA preparation are describe as before (section 2.6)(For the sequence of primers and probes used in this thesis please see Appendix Table3).

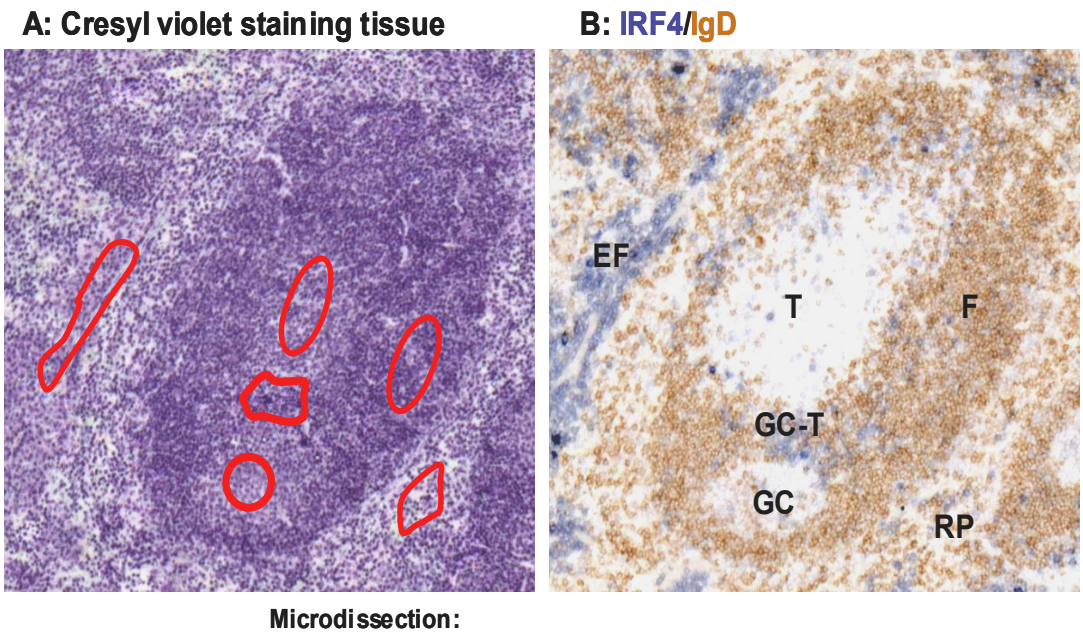

Figure 2.4: Photomicrographs of two proximate splenic sections from a mouse sacrificed 5 days after NP-CGG immunisation in carrier-primed mice demonstrating relationship between appearances after cresyl violet staining, and after immunohistochemical staining for IgD and IRF4.

A: spleen section immunohistochemically stained with cresyl violet, revealing intense staining of the B cell follicle, slightly weaker staining of the GC, T zone, and light staining of the red pulp. **B**: Junction section was stained for IgD (brown) and IRF4 (blue). This is representative of two individual experiments.

2.10 Mouse Genotype by PCR

2.10.1. Preparation of genomic DNA from mouse ear tip for PCR

About 2 mm of ear tip was placed into each of the labelled sterile 1.5 ml Eppendorfs, and 300 µl of 50 mM NaOH was added to each labelled tube. This was mixed and heated up at 98 ^{o}C for 30 minutes, and then vortexed for seconds, returned to heat at 98^{o}C for another 30 minutes or until sufficiently lysed. The mixture was neutralized with 30 ul of 1 M Tris (pH8), and mixed by vortexing again. The mixed solution was centrifuged for 6 min 13,000rpm at room temperature, and finally the supernatant (about 100µl) was transferred into a sterile 0.2 ml tubes, stored at -20^{o}C for later use.

The receipts of Solution

1M Tris (pH8)

60.6g Tris Base (Sigma T1503-5kg)

500ml ddI H_20

Autoclave

50 nM Sodium Hydroxide

2g sodium hydroxide (Fisher S318-500)

1 L ddI H_2O

Autoclave

2.10.2 PCR typing

The V region gene was amplified by PCR to check whether the daughter mice inherit the expected genes when different mouse strains cross in order to producing a right mouse model. For example, to study IgG1 switching in Cγ1-Cre mice, we use Cγ1-Cre× ROSA26 eYFP to obtain an F1 hybrid (Cγ1-Cre$^{+/-}$× R26eYFP$^{+/-}$) strain.

The PCR reaction was made with by 1µl of template, 1µl of primers (20µM), 10µl of polymerase and 7µl of water (REDtaq ready mix PCR reaction kit, Sigma UK). The solution was mixed by a pipette in an 8-well strip and centrifuged for a few seconds, run

in a Techne PCR machine, no oil but with a heated lid, calculated temperature. (Genes primer sequences see Appendix Table 1 and 2)

2.11 Statistics analysis and presentation of data

The Wilcoxon Mann-Whitney Test is a nonparametric test for assessing whether two samples of observations come from the same distribution. (Details on (Campbell 2000).We used this to compare the difference of the two independent groups. Figures presented were created by Excel 2005 and Powerpoint 2005 (Microsoft).

Chapter3 Setting the threshold - On the role of antibody for selection of Germinal Centre B cells

3.1 Introduction

3.1.1 The role of immune complex and FDCs for the selection of GC B cells

Follicular dendritic cells (FDCs) are considered to develop from stromal cells of mesenchymal origin, or from migratory precursor cells (Cyster JG 2000), or generate from the fusion of stromal cells and migratory $CD35^{+}B220^{+}$ cells (Murakami T 2007). B cells are known to be required for FDC development (MacLennan 1994). Tumour necrosis factor (TNF) and lymphotoxin (LT) are essential for their generation and development (Fu YX 1999; Tumanov AV 2003).

FDCs are present in the central region of the primary follicles, whilst they reside mainly in the LZ of secondary follicles, where they have long processes called dendrites. Some are also found in the DZ (El Shikh ME 2006). FDCs express a high level of CR1/CR2 (CD35/CD21) which bind to fragments of C3 (Imai Y 1996; Fang Y 1998). In the LZ they upregulate two low affinity Fc receptors: CD23 (FcεRII), the low affinity receptor for IgE, and FcγRIIB (CD32), the receptor for IgG (Qin D 2000). Recently identified Fc receptor for IgM, known as Fcα/μR, is predominantly expressed on FDCs, MZ B cells and follicle B cells in mouse spleen (Shibuya A 2000; Honda S 2009). Deposition of immune complex on FDCs is dependent on CR1/CR2 and Fc receptors. Monoclonal antibodies for FDC antigens such as FDC-M1 (Kosco MH 1992; Le HM 1996) and FDC-M2 (Berney C 1999; Taylor PR 2002) are used as markers for FDCs. The antigen bound by FDC-M1 is also present on tingible body macrophages. FDC-M2 binds to the complement component C4, present in immune complex. In addition, FDCs in the LZ express the adhesion molecules ICAM-1, VCAM-1 and MAdCAM-1 (Maeda K 1995;

Imai Y 1996; Szabo MC 1997; Balogh P 2002). FDCs, like other follicular stromal cells, produce CXCL13 (Cyster JG 2000). CXCL13 and its receptor CXCR5 are critical for B cell migration into follicles and are also required for B cell migration into the LZ (Allen CD 2004). SDF-1 produced by FDCs in the DZ has a crucial role in organizing GCs into LZ and DZ (Allen CD 2004).

A major role of FDCs is presentation of antigen in the form of immune complex (IC) to naïve B cells when they survey primary follicles, and to GC B cells when they test their BCRs by binding antigen (MacLennan 1994; Tew JG 1997; Allen CD 2008). Immune complexe typically contains antigen–antibody, antigen plus complement. Immune complexes are transported to FDCs by naïve B cells from the subcapsular sinus in lymph nodes (Phan TG 2007), and by MZ B cells from the MZ in spleens (Cinamon G 2008). Both types of B cells can bind ICs through CR1 and CR2.

The classic theory suggests that ICs on FDCs play a major role in selecting high affinity B cells and promoting affinity maturation in GC (MacLennan 1994). Immunization with IC can increase the repertoire of antigen-active B cells early in the response (Nie X 1997) and increase SHM (Song H 1999) within the GC. Tew's group also found that preformed ICs, which would be rapidly trapped by FDCs, stimulated SHM more efficiently than free immunogen (Wu Y 2008). Research *in vitro* demonstrated that some types of IC on FDCs can strongly stimulate B cells (Tew JG 2001) to induce expression of AID, leading to SHM and CSR, finally promoting the production of high affinity Ig (Aydar Y 2005). TD antigens in IC on FDC can cross-link B cell receptors and trigger a TI like immune response (El Shikh ME 2009). In addition, recently *in vitro* and *in vivo* studies suggested that TLR4 signalling modulates FDC activation and maturation, strongly impacting on SHM and the generation of Ig class switched high affinity B cells (El Shikh ME 2007; Garin A 2010). These findings further support the regulatory role of IC-bearing FDCs on

affinity maturation. IC deposition on FDCs may have a role for the differentiation of GC B cells into plasma cells or memory B cells. As the immune response progresses, antigen-specific IgG will be produced. Engagement of FcγRIIB on GC B cells by IgG-ICs and altered engagement of BCR may generate a signal that stimulates centrocytes differentiation into memory B cells (Tarlinton DM 2000).

3.1.2 Mathematical modelling GC reaction

Mathematical modelling of GCs using a combination of state charts and cellular automata can create a 3D interaction environment where chemotaxis, cell migration, cell division and apoptosis, cell contact and interaction and cell differentiation can take place *in-silico*. Each cell type and physical object is assigned a digital code to describe the cellular activation (Iber D 2002; Meyer-Hermann 2002). Mathematical modelling provides a quantitative and systematic framework for the verification of hypotheses and theories on the GC reaction, and adds a different vantage point to experimental data. For example, mathematical modelling of the GC indicated that soluble antibody may have a role in the selection of high affinity B cell clones, and therefore affinity maturation (Iber 2002). A theoretical study by Meyer-Hermann (2006) leads to the hypothesis that competition for helps from T_{FH} cells in GCs is an efficient mechanism for achieving the selection of high affinity clones (Meyer-Hermann ME 2006; Allen CD 2007b).

A recently published study used mathematical modelling of the GC reaction with cell-based space-resolved methods to reinterpret motility data generated from experiments using intravital two-photon microscopy (Figge MT 2008; Meyer-Hermann ME 2009). The mathematical analysis found that although GC B cells move via a random walk, zones of organisation can be maintained by transient chemotaxis. This is dependent on periodic sensitivity and desensitisation of B cells for the chemokines CXCL12 and/or

CXCL13 (Figge MT 2008). Therefore, *In-silico* experiments can help to identify critical time points and experimental conditions for conclusive *in vivo* experiments (Figge MT 2008, Garin A 2010).

In GCs, B cells proliferate and hypermutate their Ig variable region genes. After this, B cells must undergo selection for specificity and affinity of their B cell receptor. This is thought to happen by interaction with antigen deposited as IC on FDCs (MacLennan IC 1997a). As antigen on FDC is present in the form of IC, antibody covering the antigen interferes with B cell antigen binding.

In this thesis I am testing the competition model that GC B cells are competing with antibody bound on antigen in ICs (Fig 3.1). During the initial phase of an antibody response this antibody will be natural antibody or low affinity antibody produced by early plasmablasts that have developed in the early extrafollicular response. Therefore initially even relatively low affinity centrocytes should be able to compete for access to the antigen. However, during the course of an immune response, this antibody may be replaced by high affinity antibody produced by plasma cells that are derived from GCs, resulting in an increase of antibody affinity in ICs (Tew JG 1997). This may also have some impact for the interaction of BCR, and centrocyte selection in the GC as well. *In silico* modelling of the GC response using GC B cells, FDCs and T cells moving and interacting on a 3D lattice over two weeks confirmed that a dynamic selection threshold, which is dependent on the affinity of antibody, is more efficient in producing output cells rapidly and at higher affinity compared to a fixed threshold model (Adam Reynold's thesis 2010, and Michael Meyer-Hermann, personal communication).

The Fig 3.1 presents migration and differentiation of GC B cells and the competition model on selection of GC B cells that was tested in this chapter. Activated B cells move

into the follicle and form proliferating centroblasts. Centroblasts proliferate and hypermutate in the DZ, after which they differentiate into centrocytes in the LZ. Here they test their mutated BCR by binding antigen on FDC. Antigen is covered with antibody as IC present on FDC, and so centrocytes have to compete with this antibody to gain access to the antigen. This antibody could provide a threshold, as only B cells with higher affinity than antibody already present may be able to gain access to the antigen and obtain positive selection signals, whilst others will die by apoptosis. The selected centrocytes move back to the DZ to further mutate, or differentiate into plasma cells, which produce higher affinity antibody. The question is whether this newly formed antibody will move back into the GC to replace the initial low affinity antibody in ICs, a process that may increase the selection threshold for subsequent centrocyte selection.

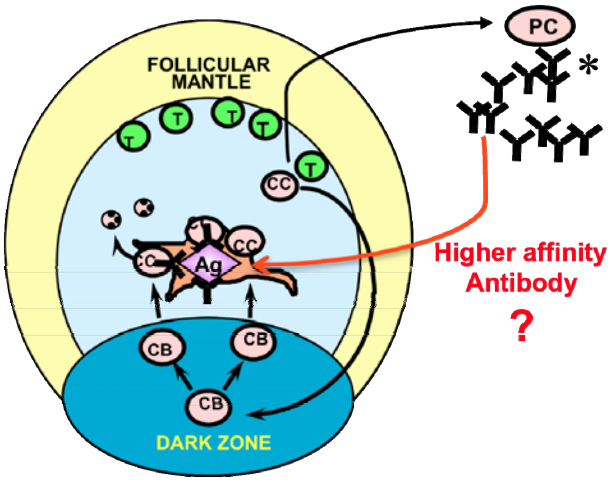

Figure 3.1: Germinal centre selection hypothesis: how antibody feedback may produce a dynamic selection threshold
Antibody produced by the GC output binds to antigen on FDC raising the selection threshold for subsequent centrocytes. As the reaction progresses the antibody held on FDC and therefore the threshold for B cells to access antigen may increase. CB: Centroblast, CC: Centrocyte, T: Follicular T helper cell, Ag: antigen, PC: Plasma cell.

The aim of this project is to test whether competition of IC antibody with GC B cells has a role in the development and selection of high affinity B cells in GCs. The detailed aims of the project are the following:

1. Are antibodies inside GCs replaced by antibodies produced outside GC?
2. Is the affinity of the antibody immobilized on the FDC important for antibody replacement?
3. Does antibody affinity have an impact on centrocyte selection?

The main approaches used include: immunohistology to investigate the appearance and disappearance of ICs, GC development and the production of early plasmablasts, and ELISA to detect the antibody affinity in sera after immunization with different affinity IgM-IC.

3.2 Results

3.2.1. Characterisation of the avidity of anti-NP clones

To provide a tool to be able to test the role of antibody affinity on the selection of B cells in germinal centres, several NP-specific hybridoma cell lines producing monoclonal anti-NP antibodies with different affinities were developed by immunization of QM, B1-8 and B1-8^{high} mice with NP-Ficoll (Detail see section 2.3.1).

Antibody binding can be defined by affinity or avidity. As the *in vivo* antigen-binding characteristic of our antibodies is determined by the whole antibody molecule, we sought to determine avidity, and not affinity, which is determined by the binding of one F_{ab} segment to one epitope. To do this, antibodies were characterised by using surface plasmon resonance. This involves measuring their binding to NP_{15}-BSA which should have a similar epitope density to NP_{15}-CGG, used in the *in vivo* experiments. This method allows analysis of the direct kinetics of binding interactions in the real time (Fig 3.2) (See method review (Adamczyk M 2001).

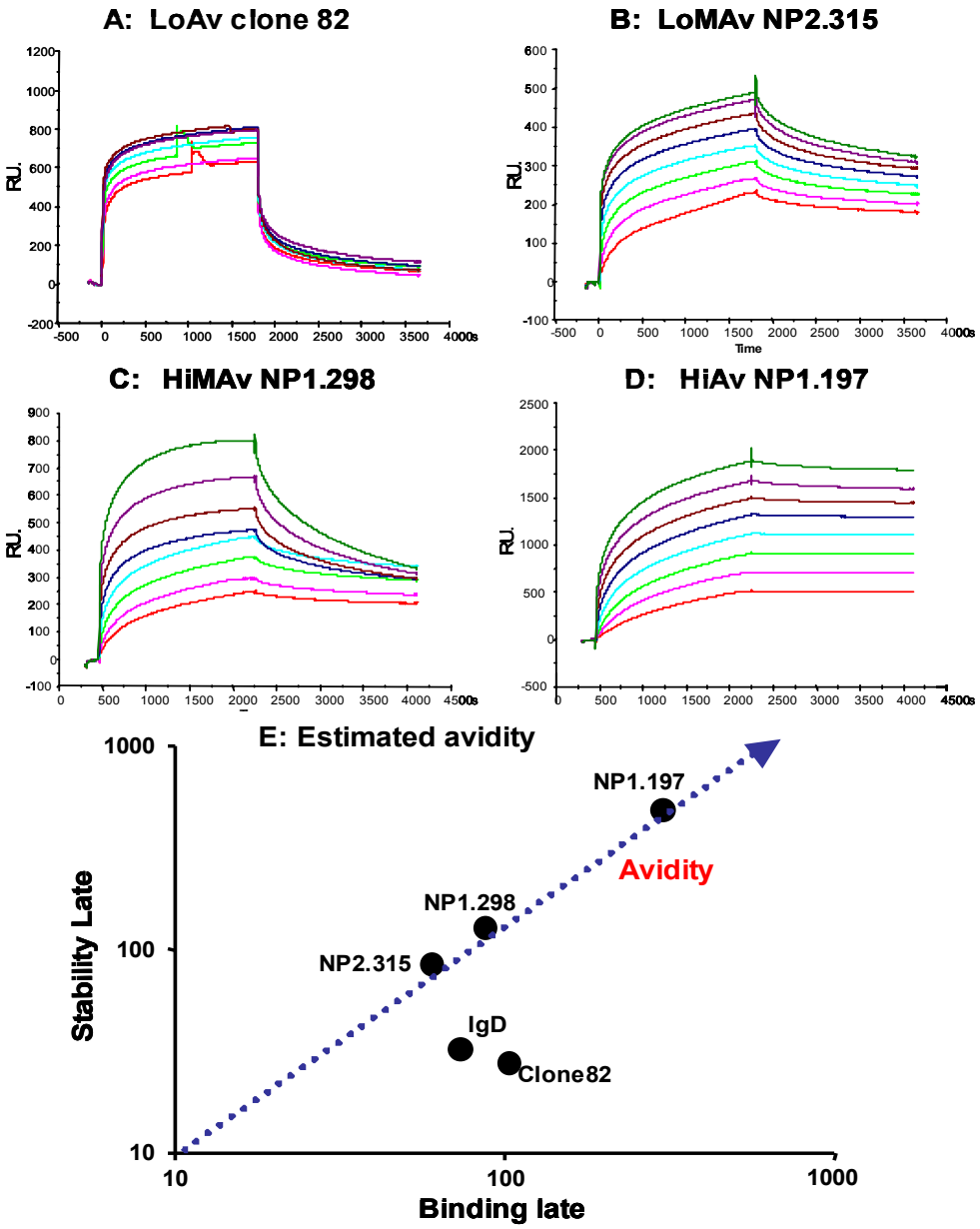

Figure 3.2: High and low avidity NP-specific IgM Clones

Surface plasmon resonance studies show the NP-specific association and dissociation of different antibody clones and the estimated avidity. (**A**) Clone82 (developed from QM) has a very fast off rate and low avidity when binding NP antigen. (**B**) NP2.315 (B1-8) and (**C**) NP1.298 (B1-8high) have similar association rates, but NP1.298 dissociates considerably slower and has a higher avidity at higher dilution. (**D**) NP1.197 (B1-8high) binds with very high avidity. Different lines show different concentrations: from 2mM,double dilutions to 15.625µM (Green line to red line). (**E**): Estimated avidity rises mainly with stability (slow off rate). Arrow shows an estimated measure of relative avidity. RU: Response unit.

Fig 3.2 shows marked differences in the binding kinetics of these four antibodies at 38^{o}C over concentrations ranging from 15.625μM to 2mM in PBS. The data are plotted on a linear scale to show differences in avidity more clearly. To measure early and late binding, response units were measured at set time points (2500 seconds after antibody injection for "stability late" and at 1500 seconds after injection for "binding late") at the lowest antibody dilution of 15.625μM. The antibody concentrations injected into mice are much lower than this, for example, 180μg or 90μg of antibody per mouse as used in our experiments would dilute to 2×10^{-4} and 1×10^{-4} mM, respectively. Stability late and binding late provide a good estimate for the relative avidity of all antibodies since the binding-late response is related to the association rate of interaction (on-rate), and stability–late is inversely related to the dissociation rate (off-rate) (BIACORE 3000 tech-note) (Fig 3.2E). Batista *et al* (1998) showed that the off-rate has a major role for determining the affinity of BCR/antigen. Anti-NP clone 82 was found to have a high on-rate, but also a very high off-rate (Fig. 3.2A), and therefore was classed as having low avidity for NP (labelled LoAv). NP1.198 (Fig 3.2 C) has a slightly higher avidity than NP 2.315 (Fig 3.2 B). NP1.198 was classed as intermediate high avidity (HiMAv), and NP2.315 was classed as intermediate low avidity (LoMAv). NP1.197 (Fig 3.2 D) has the slowest off-rate, and was used as high avidity NP-specific IgM^{a} (HiAv) in further experiments.

3.2.2. The appearance of injected IgM^{a} immune complex in germinal centers

3.2.2.1 Experiment design

Experiments were carried out on mice that express endogenous IgM^{b} allotype in order to test the competition model, that antibody inside GC would be replaced by antibodies produced by GC output cells during an immune response. Mice had been primed with CGG, and were then injected with immune complex of NP-CGG plus anti-NP IgM^{a},

which is a low avidity antibody clone82 (LoAv). Carrier priming induces GC development within three days by accelerating B cell activation through immediate availability of T cell help (Toellner KM 1996). It may also reduce the threshold for B cell activation by antigen opsonisation by preforward carrier-specific antibody (Sze DM 2000; Sze DM-Y 2002). Immune complex, containing 20μg of NP-CGG, was prepared with a ratio of hapten/antibody antigen-binding site of 1, and mixed well 30 min before *i.v.* injection. For 5 days after immunization the replacement of IgM^a with IgM^b in the IC on FDCs, and the GC response were followed (Fig 3.3).

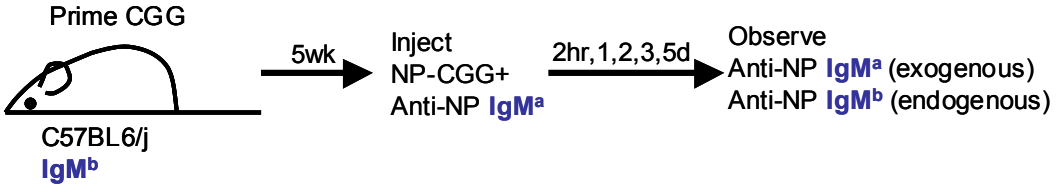

Figure 3.3: Protocol for the experiment on appearance and disappeaance of antibody from germinal centre
C57BL/6 mice were primed with CGG. 5 weeks later, mice were injected with immune complex of NP-CGG+ anti-NP IgM^a, the apperance and disapperance of IgM^a and IgM^b from the GC were followed over the next 5 days.

3.2.2.2 Optimisation of IgM^a and IgM^b

IgM^a and IgM^b antibodies were selected to identify the extraneous IC and endogenous antibodies in the spleen sections. In preliminary experiments antibody dilutions for immunohistochemical staining were optimized. Furthermore, the absence of cross-reactivity to the other allotypes was tested by staining splenic tissue from mice with allotype IgM^a or IgM^b.

Frozen spleen sections from IC immunized mice were stained for IgM allotype and IgD. GCs were identified as IgD^- areas surrounded by an IgD^+ follicular mantle. As seen in fig 3.4A, in non-immunised BALB/c mice (allotype IgH^a) IgM^a is detected clearly on all non-switched B cells and on plasma cells in the extrafollicular foci. IgM^b was not detectable on others sections from the same animal (Fig 3.4C).

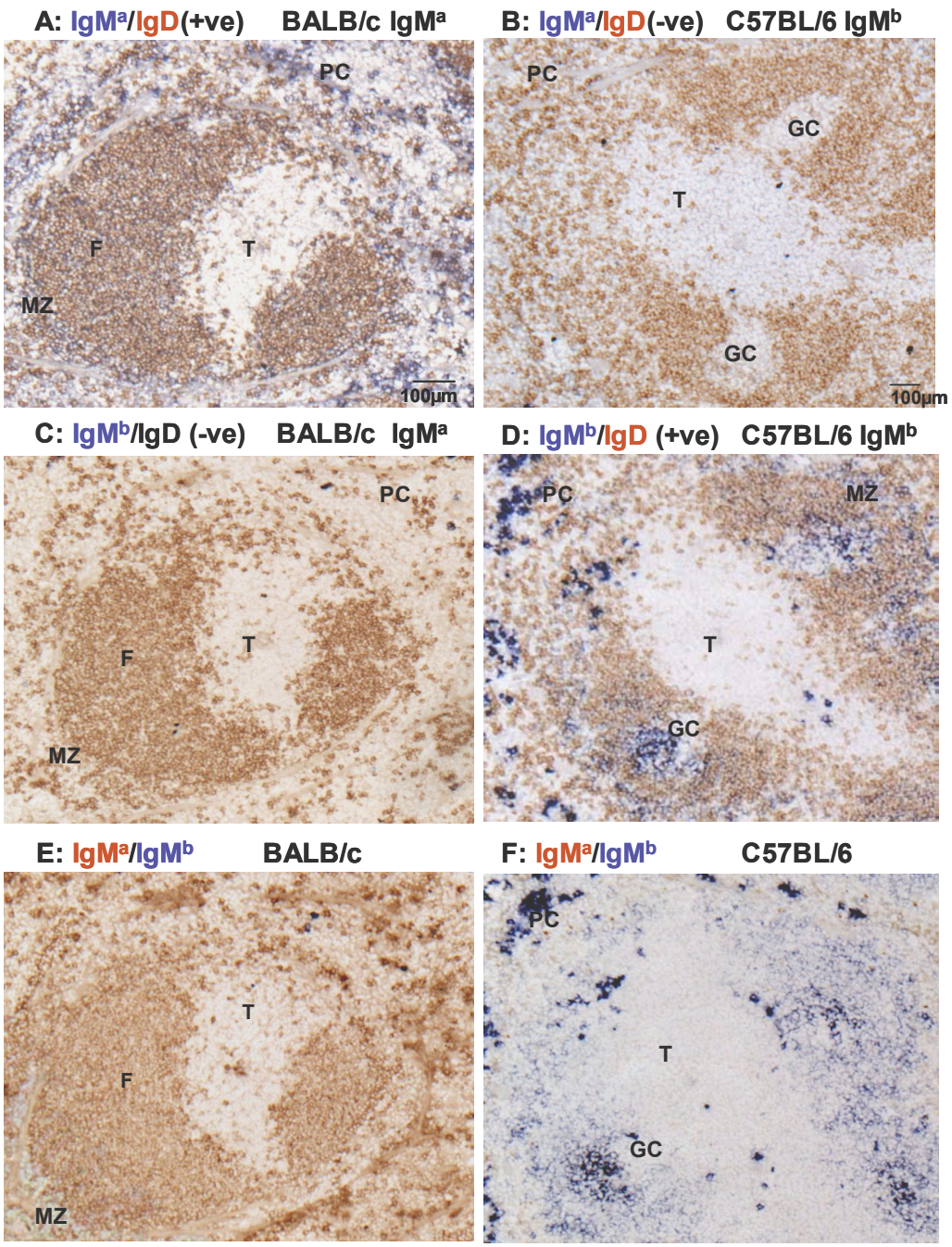

Figure 3.4: Optimisation of the IgM^a/IgM^b specific immunohistology

A: Spleen sections from BALB/c mice (allotype IgHa) as IgMa positive, **B**: spleen section from C57BL/6 mice (IgHb) as IgMa negative control. **C**: BALB/c spleen tissue as IgMb negative control, and **D**: C57BL/6 spleen as IgMb positive control. E: IgMa (brown) IgMb (blue) staining on BALB/c spleen tissue. F: IgMa (brown) IgMb (blue) staining on C57BL/6 spleen tissue. T: T zone; GC: germinal centre; F: follicle; PC: plasma cells, MZ: Marginal zone.

In C57BL/6 mice, IgM^a-specific antibodies did not produce any non-specific staining (Fig3.4 B), while IgM^b was detectable in a similar pattern to IgM^a in BALB/c mice. After immunisation of C57BL/6 mice IgM^b was strongly positive on plasma cells, FDC network, non-switched GC B cells and MZ and follicular B cells (on Fig 3.4D). Double immunohistochemical staining for IgM^a and IgM^b of BALB/c or C57BL/6 spleen sections showed only the respectively expressed IgM allotype, while the other one was undetectable or showed only weak non-specific cross-reaction in the red pulp (Fig3.4 E F).

3.2.2.3 Appearance of IgM^a immune complex in Germinal Centres

Immunohistological staining was used to detect the appearance and disappearance of IgM^a antibody in IC on spleen sections at different time points. Four serial cryostat sections were stained to detect NP-binding cells with IgD, IgM^b with IgD, IgM^a with IgD, and IgM^a with IgM^b. Fig 3.5A clearly shows the background without any non-specific IgM^a staining in the follicle of non-immunised mice. IgM^a-IC arrives in the MZ and is rapidly transported towards the follicle within 2 hours of injection (Fig 3.5B). One day after immunization all IgM^a-IC localizes on FDC in the GC light zone (Fig 3.5C). Immunofluorescent staining using FDC-M2 and FDC-M1 to identify FDC (Kosco MH 1992; Taylor PR 2002) confirms that IgM^a-IC is mainly located on FDC (Fig 3.5D, E), and not on GC B cells (Fig 3.5 D-F).

Three days after injection, the injected IgM^a antibodies start to disappear from the FDC network (Fig 3.6A), and at day 5, IgM^a is hardly detectable in the GCs (Fig 3.6B). At the same time staining for endogenous IgM^b shows that FDCs still display much IC that is covered with IgM^b antibody with a constant density at both day 3 and day 5 (Fig 3.6C, D). NP-specific plasma cells and GC B cells were measured by incubating sections with sheep-Ig that had been conjugated with NP. At day 3, only occasional antigen-specific

(NP-binding) B cells are detectable in follicles and the red pulp (Fig3.6E). These expand in the red pulp within the next two days to form early extrafollicular plasma cells (Toellner KM 1996, Sze-DM 2000), or form large GCs filled with NP-specific GC B cells (Fig. 3.6F). Comparing IgM^a, IgM^b and NP-specific staining show that much of the IgM^b staining in the GC is due to the presence of NP-specific IC on FDCs, which seems to be produced by early extrafollicular plasmablasts. The disappearance of IgM^a is likely to be due to replacement by endogenous NP-specific IgM^b, not due to the disappearance of NP-specific IC.

3.2.2.4 Quantifying the disappearance of immune complex during an antibody response

To measure the appearance and disappearance of IgM^a-IC in GCs, we used the ImageJ software to quantify the DAB derived brown and the Fastblue derived blue staining of IC on the FDC areas (detailed method see section 2.6). One or two areas of interest per GC and in total 10-20 regions of interest per individual tissue section were chosen. Median intensities of IgM^a and IgM^b staining were quantified from different areas from one spleen tissue. Median values from one spleen section at an individual time point are shown in Fig 3.7 A and B. Each diamond represents one region of interest in the GC.

Immunoenzymatic staining is not ideal for quantifying the presence of antigen densities on tissue sections, as staining intensity depends on the timing of enzymatic colour development. Furthermore, dye deposition can become too dense for bright-field microscopic quantification, and brown and blue dyes have considerable spectral overlap. We tried to control this by developing tissue sections at the same time, avoiding overdevelopment by microscopic control. Further control experiments were done with quantitation of fluorescent staining. These produced similar results, but were of inferior sensitivity.

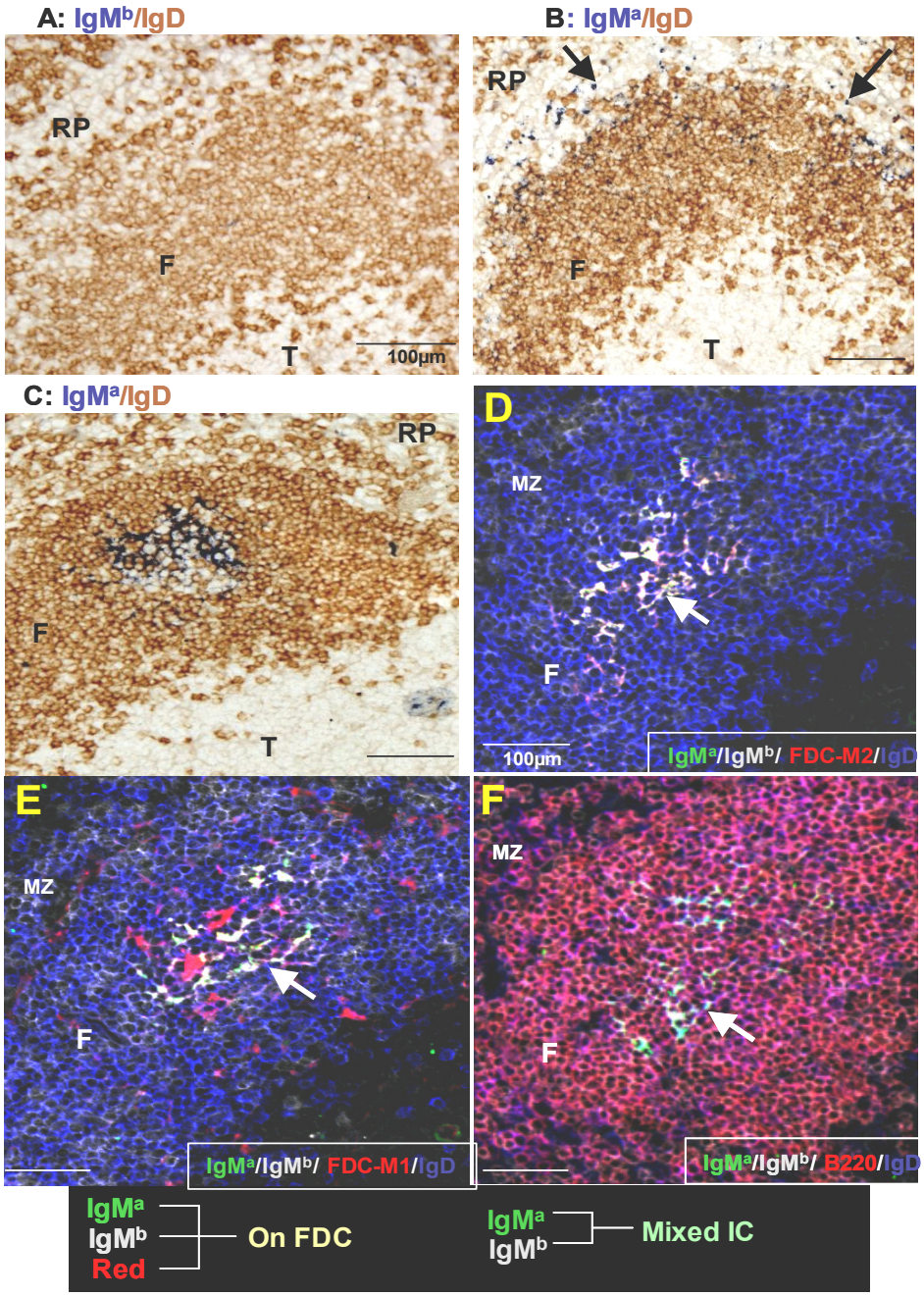

Figure 3.5: IgM^a immune complex appearing on FDC

Low avidity NP-specific IgMa antibody (clone 82)– antigen IC was i.v. injected into CGG primed mice. **A**: white pulp area (IgD$^+$) section before injection, **B**: IgMa-IC transported toward follicle from marginal zone 2hr after injection (arrow), **C**: IgMa-IC localized in the FDC area at 1d after injection. Fluorescent staining by using FDC-M2 (**D**) and FDC-M1(**E**), B220 (**F**) confirms IC sitting on FDCs (white arrow) F: Follicle;GC: Germinal centre; T: T zone, RP: Red Pulp, MZ: Marginal zone

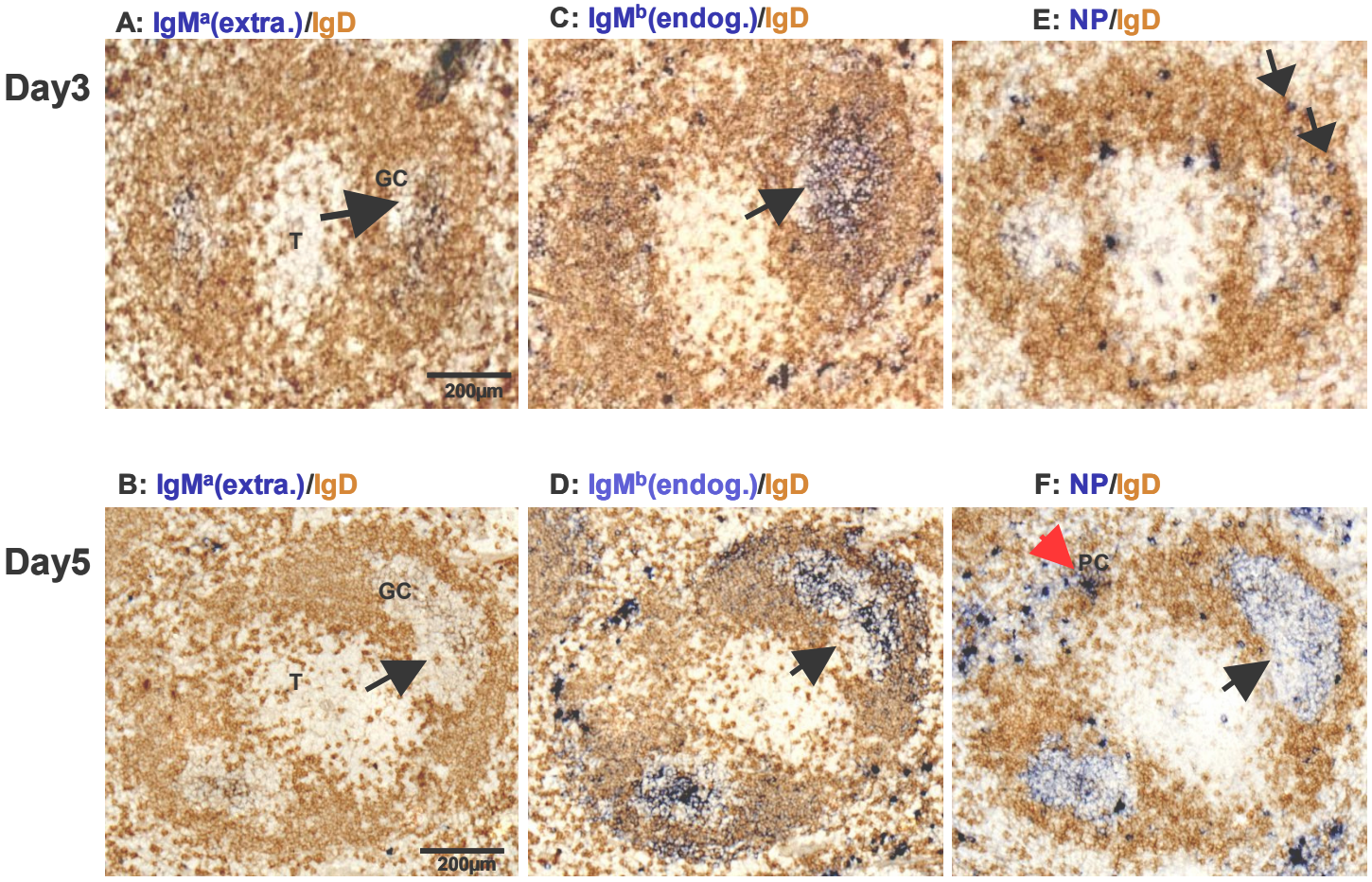

Figure 3.6: Disappearance of exogenous IgM^a

Three days after injection IgMa-IC starts to disappear from FDCs (**A**), at day 5, IgMa-IC is hard to detect (**B**), while staining for endogenous IgMb shows that FDC still display much IC day3, 5 after immunization (**C. D**). Antigen-specific (NP$^+$) B cells are first detectable in GC on day 3 (**E**). On day 5 considerable numbers of plasma cells in the red pulp produce NP-specific antibody, and big NP+ GC forms (**F**). GC: Germinal centre, T: T zone, PC: Plasma cell.

This method always produces some brown and blue background detected on stained sections even though only one antigen was present and only one colour was seen visually. Therefore positive control areas were taken that have mainly IgM^b-specific FastBlue staining, i.e IgM^b expressing plasma cells in the extrafollicular regions, and areas that have maximum DAB derived IgM^a-specific brown staining and contain minimal amounts of endogenous Ig, i.e. MZ macrophages containing IgM^a-IC shortly after injection. These are labelled brown and blue on Fig 3.7 C, D. IgM^a (blue) and IgM^b (blue) median densities from the same GC areas of one section at the different time points are shown in Fig 3.7 A and 3.7 B respectively. Ratios of IgM^a (brown)/IgM^b (blue) densities from double staining of IgM^a/IgM^b on the same GC following the time point are shown on Fig 3.7 C. Data from different animals are summarized in Fig 3.7 D, showing that the results from different animals were highly reproducible.

The figure shows the arrival of IgM^a in the MZ and its binding to phagocytes within 2 hr (brown diamond). At the same time a clear increase above background level of IgM^a is detectable on FDC. IgM^a on FDC peaks 1 day after immunization. At the same time IgM^b on FDC seems to be displaced. This probably shows that there is a limit to the amount of immune complex that can be stored on FDC. Over the next 4 days IgM^a levels on FDC decrease and IgM^b levels rise again. The IgM^a staining on FDC is not significant although there is a slight trend to higher levels on day 5 than on day 0. These observations are compatible with the hypothesis that extraneous IgM^a is deposited on FDC in GCs. Over the next few days this disappears due to replacement by IgM^b produced by the early extrafollicular plasma cells.

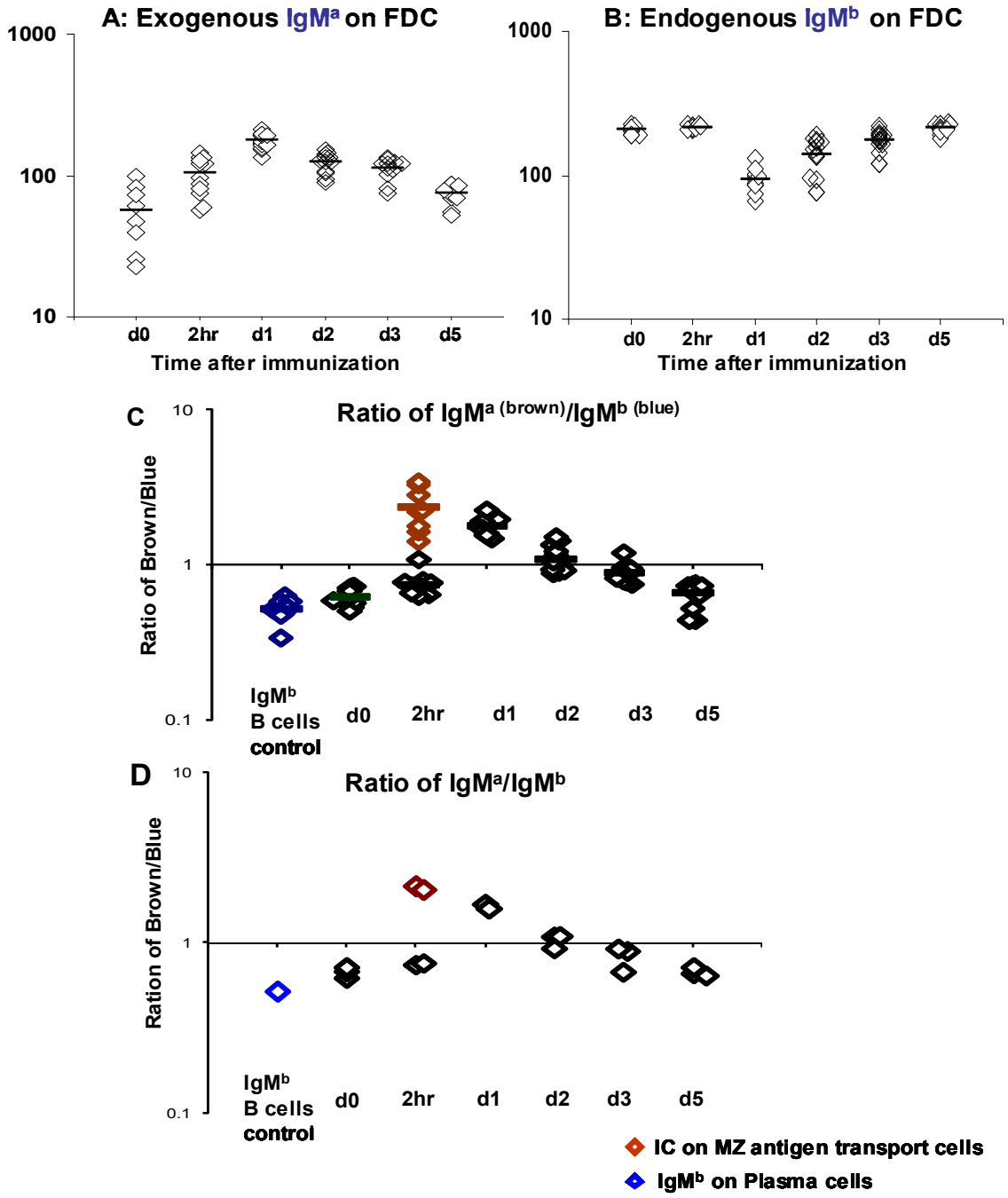

Figure 3.7: Quantification of relative amount of IgM^a and IgM^b

The relative amount of IgMa (blue) and IgMb (blue) of the same interest region in GC from one spleen section at different time point are respectively shown on (**A**) and (**B**) (Each diamond represents one area interest in GC). Then calculate the ratio of IgMa(brown)/IgMb(blue) was calculated from the same area of interest. **C**: Each diamond indicates ratio of IgMa/IgMb for one FDC, all data from 1 spleen; **D**:Each diamond represents average of ratio from one mouse.

3.2.3 Impact of antibody avidity in immune complex for B cell response

Next we decided to investigate what the effect of antibody avidity in IC present on FDC has for the kinetics of the replacement of antibodies in ICs on FDC. Further, the effects of antibody on selection and affinity development in GC were studied. To do this we studied GC output, serum antibody affinity, GC size and lifespan. Mice, primed with CGG 5 wk earlier, were challenged with 20μg immune complex of NP-CGG plus anti-NP IgM^a of intermediate low or intermediate high avidity via *i.v.* injection. The LoMAv (NP2.315) and HiAv (NP1.198) IgM^a antibody were developed from B1-8 or B1-8^{high}, as described earlier (section 3.2.1).

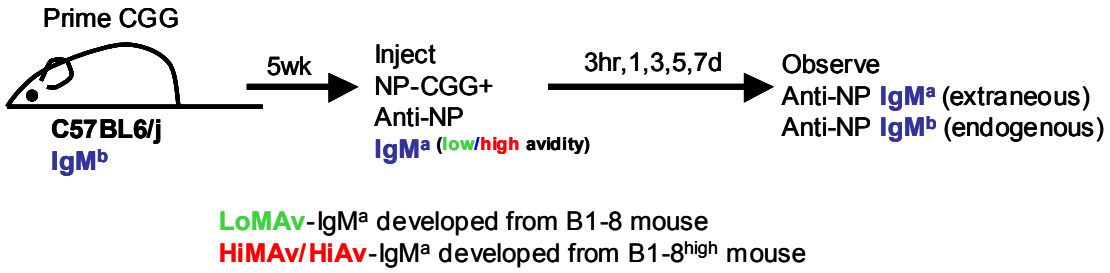

Figure 3.8: Experiment to detect impact of different antibody avidity in IC for B cell development and selection in GC

3.2.3.1 Intermediate high avidity antibody (HiMAv)

Initially, LoMAv (NP2.315) and HiMAv (NP1.298) were selected. The appearance and disappearance of IgM^a-IC in GCs were observed on cryostat spleen sections by immunoenzymatic staining. Interestingly, five days after injection of ICs containing LoMAv or HiMAv antibody, LoMAv IgM^a-IC had almost disappeared from GCs (Fig 3.9A), while at the same time the higher avidity antibody was still detectable in GCs (Fig3. 9B). The quantification of the size of IgM^a-containing FDC area in the whole GCs shows a significant difference between the two groups at day5 with more of the higher avidity IgM^a-IC detectable (Fig 3.9E). A similar trend was seen on day 7, although this was not significant because of the small number of animals used. This observation of

more HiMAv IgM^a-IC seen in GCs at d5 was reproduced in a second independent experiment. Big NP specific GCs were formed and high numbers of antigen-specific plasma cells were produced in the red pulps in both groups at d5 (Fig 3.9 C D). In the LoMAv group, IgM^a-IC disappeared, and may be replaced by newly formed antibody from the GC output.

It appears that the antibody avidity determines the kinetics of antibody replacement in GCs. Therefore we then assessed whether antibody avidity in IC affects GC B cell selection by quantifying the number of apoptotic cells in the GC. Caspase-3 is a member of the cysteine protease family, which plays a crucial role in executing apoptotic pathways by cleaving a variety of key cellular proteins. Antibodies to activated caspase-3 can be used as a marker for cells that have entered apoptosis irreversibly (Devarajan E 2002).

Apoptotic cells in the GC were assessed on d5 and d7 when the GC reached a significant size. Apoptotic cells were found in GCs (Fig 3.10 A) and were also present in the red pulp (data not shown). Although the frequency of apoptotic cells in the samples at day5 after injection is quite variable, interestingly, GCs from the mice that received HiMAv IC already had more apoptotic cells ($p=0.025$). This difference trend was apparent at day 7. Because of the small group size on day 7 this difference was not significant ($p=0.2$) (Fig 3.10 B). According to our competition model centrocytes are expected to enter apoptosis if they fail to be selected because their BCR affinity is lower than the antibody affinity on FDCs. Higher frequencies of apoptotic cells in GC developing after immunisation with HiMAv IC are compatible with our model that centrocytes are in competition with antibodies in ICs on FDCs, and this is mainly dependent on antibody avidity for the antigen.

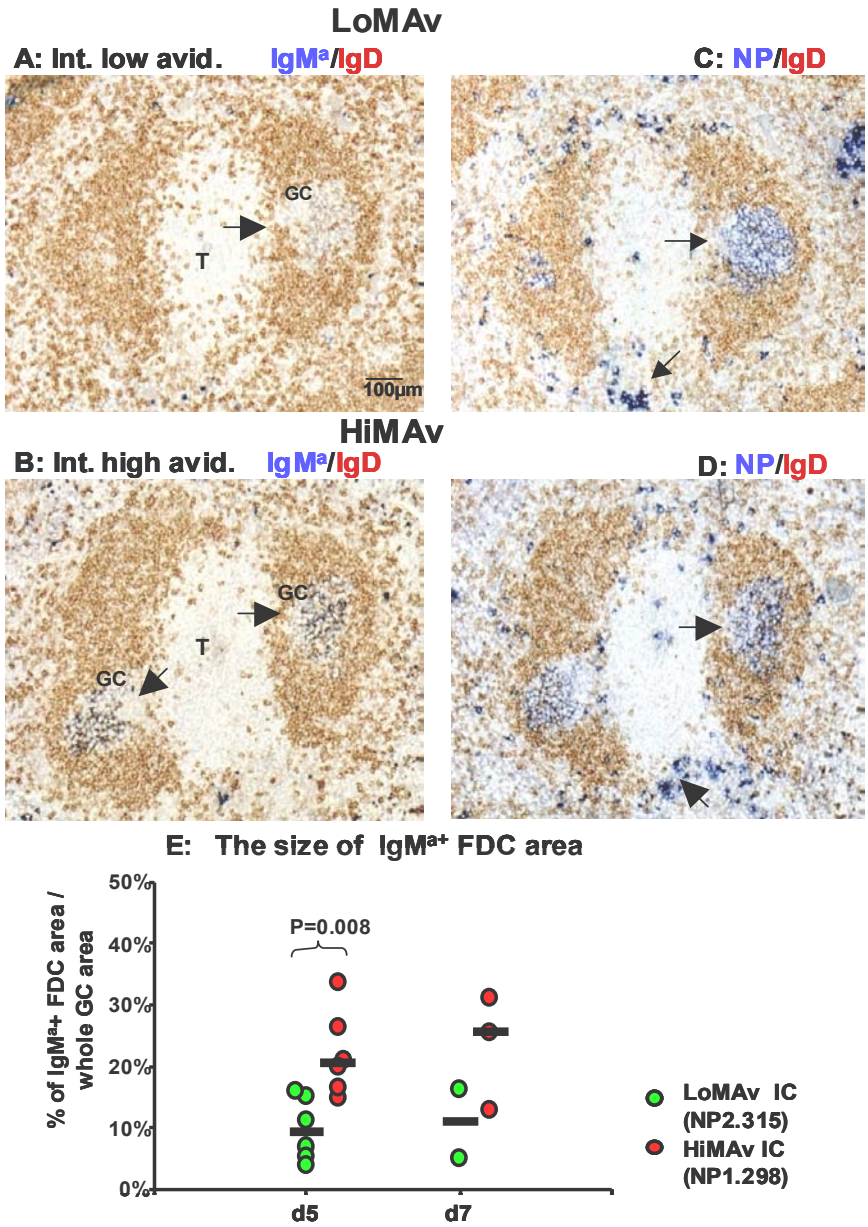

Figure 3.9: The effect of avidity on the replacement of antibody in immune complexes on FDC.

CGG primed mice were injected with NP-CGG plus anti-NP IgMa of LoMAv or HiMAv antibody. 5 days after injection LoMAv IgMa had almost disappeared (**A**), while HiMAv IgMa-IC is still easily detectable in GC (**B**). Antigen specific (NP^+) plasma cells in the red pulp produce the antibody and large NP^+ GCs formed. There is no significant difference in the amount of NP staining in GC induced with higher or lower avidity IC (**C.D**). (**E**): As a measure for the total amount of injected antibody at the different times after immunisation, the percentage of $IgMa^+$ FDC network of the whole GC area was measured at d5 and 7 after injection. Each points represents one mouse. $p<0.001$ between int. low and int. high avidity, according to the Wilcoxon Mann-Whitney U test. This is representative of two individual experiments. LoMAv: intermediate low avidity (NP2.315), HiMAv: intermediate high avidity (NP1.298).

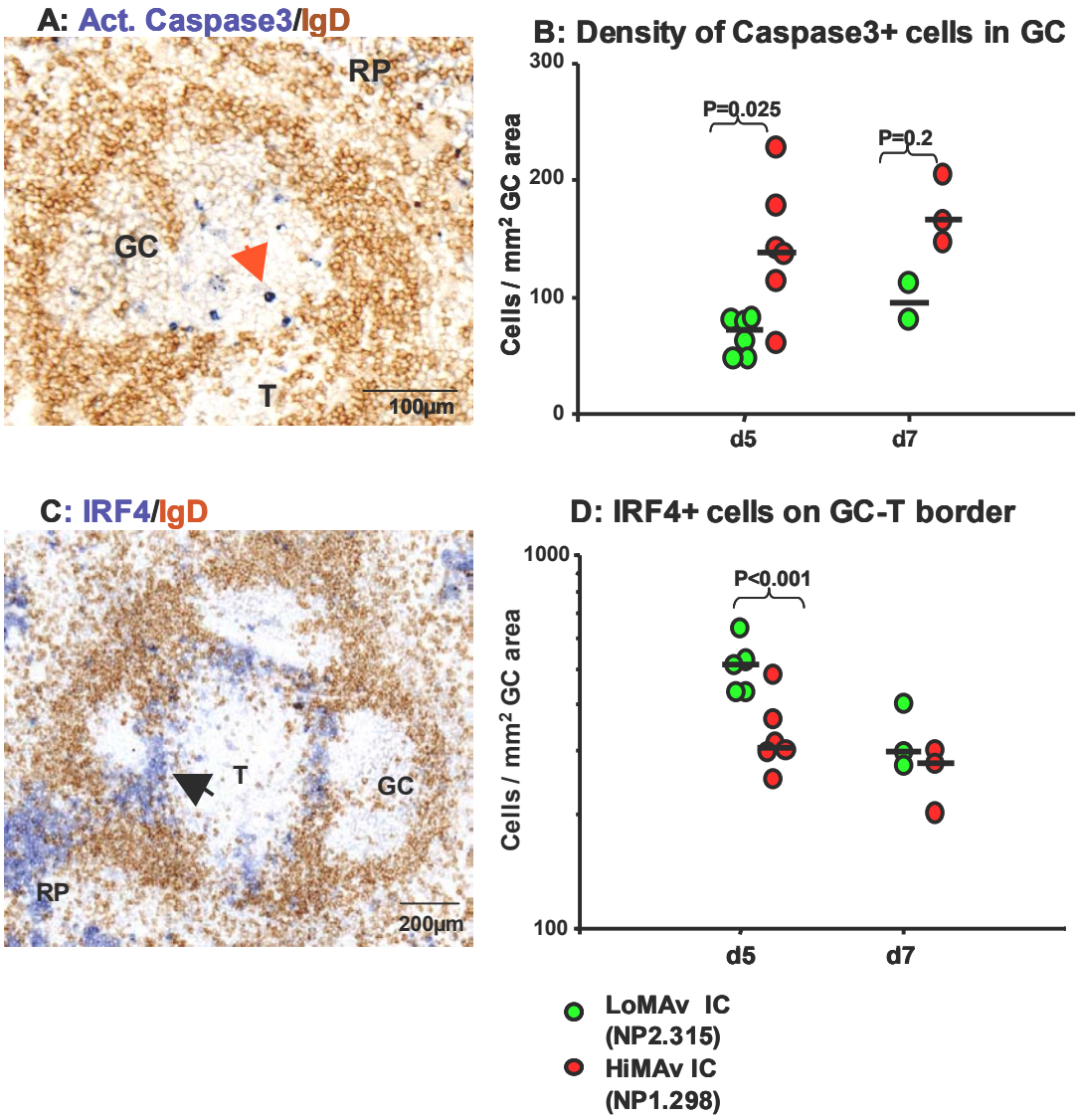

Figure 3.10: Effect of antibody affinity for Caspase3^{+} apoptotic cells in GC, and GC outputs.

The number of apoptotic cells was assessed by staining caspase3 at d5 and d7 after immunisation with LoMAv/HiMAv IgMa-IC. **A**: Caspase3 histological staining in tissue section. (**B**) Density of Caspase3+ apoptotic cells in GC area.**C**: IRF4 as marker for the early GC output was used to stain on tissue section. **D**: Density of IRF4+ cells on the GC-T zone border. Number of cells is expressed as the number per mm2 of GC area on tissue section. Each point represents one mouse. $p<0.05$ between LoMAv and HiMAv group, according to the Wilcoxon Mann-Whitney U test. This is one of two individual experiments. GC: germinal centre; T: T zone; RP: red pulp. LoMAv: intermediate low avidity (NP2.315), HiMAv: intermediate high avidity (NP1.298).IRF4, a transcriptional regulator, has been suggested to act upstream of (Sciammas R 2006) or in parallel to

(Klein U 2006) Blimp1 in the differentiation of plasma cells. IRF4 mRNA peaks in B cells within 1h after immunization with a TI antigen, and IRF4 protein expression also becomes detectable within 1h. Therefore, IRF4 can be used as a marker induced immediately after activation of B cells (discussed in chapter4). IRF4 expression is lost when B blasts move into follicles and differentiate into GC B cells (Cattoretti G 2006a), Marshall, Zhang *et al*, manuscript in preparation), but regain as soon as GC B cells leave the GC and form plasma cells. These cells are detected in the GC DZ-T zone interface (discussed in chapter4). We used IRF4 expression in this area to detect the plasma cell output from GCs. Interestingly, more IRF4 plasmablasts were found on the GC-T zone interface at day5 after NP-CGG immunization in the carrier-primed mice (Fig 3.10 C). These cells are plasmablasts exiting GCs, as they express plasma cell markers such as CD138, and antigen-specific Ig, and at the same time downregulate GC markers such as GL-7, Bcl-6, and PNA (Further details about IRF4 are discussed in Chapter 4).

GCs were seen in tissue sections at 5days post immunization. High numbers of $IRF4^+$ cells were present at the GC-T zone interface, and significantly more cells on the interface when mice received LoMAv IgM^a-IC than another group, which were immunized with HiMAv IC ($p<0.001$) (Fig 3.10D). At day7, the number of $IRF4^+$ plasmablasts at the GC–T zone interface was lower compared to day5. No significant difference was detected between the two groups. This result again reflects the effects on the selection threshold in GCs. This data supports the competition model, that higher avidity antibody results in a higher selection threshold. Therefore, in the presence of higher avidity antibody, less B cells will pass the strict selection process and then differentiate into the plasmablast output. Centrocytes may undergo several cycles of proliferation and somatic hypermutation to develop competitive BCRs that can compete with the high avidity IC deposition on FDCs. Therefore, effects on the selection from HiMAv IC immunization will be higher on day5, just 2d after GCs have formed, then on

day7.

Mice produce IgM as the primary Ab, followed by production of IgG after Ig class switching. ELISA was used to detect the production of new NP-specific serum antibody of different classes after immunization. As the IC injected contains IgM^a, only IgM^b was measured to detect endogenous IgM production. An increase above detection level was first seen at 5d after injection with IC (Fig 3.11A). This is because newly formed plasmablasts do not start antibody production before day3 after immunization (Sze-DM 2000). There is no significant change in the level of IgM^b production between mice which got HiMAv and LoMAv IC group.

NP-specific IgG titre and affinity were tested by detecting antibody on NP_{15}-BSA and NP_2-BSA coated ELISA plates, and comparing the levels. IgG above detection level was first observed at day5 after injection with IC. While no significant difference between LoMAv and HiMAv in total IgG production was detected (Fig 3.11B), mice challenged with HiMAv IC produced higher affinity IgG ($p=0.004$). At day 7 the difference between the groups became smaller with both producing high affinity antibody (Fig 3.11 C.D). HiAv IC induced the earlier onset of high affinity antibody production, due to increased competition at the early stages of the GC response. Independent *in silico* modelling produced similar results, with an earlier onset of high affinity GC output (unpublished, see 3.3.6 Discussion). The difference between LoMAv and HiMAv becomes smaller later in the response. This may be due to GCs induced by LoMAv IC adapting a similar threshold later in the response, after higher affinity plasma cells have left these GCs and produce antibodies.

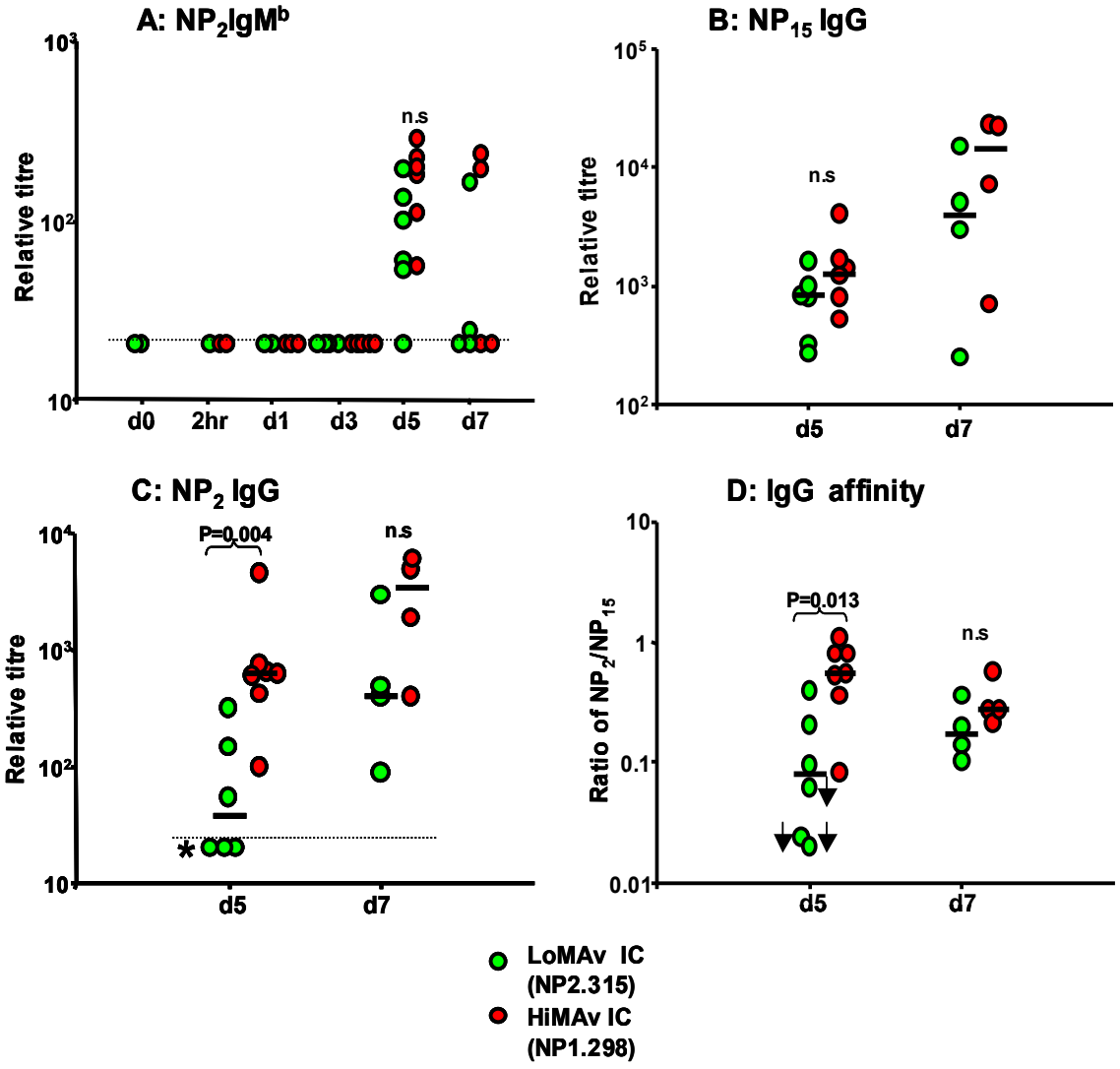

Figure 3.11: Endogenous IgM^b and IgG titre after injection of LoMAv and HiAv IC

The figure shows the relative IgM^b and IgG titre at 5 and 7 day after 2nd injection with LoMAv/HiMAv IgM^a-IC. Each spot represents one mouse. **A**: (NP_2) IgM^b titres; **B**: (NP_{15}) IgG titres; **C**: (NP_2) IgG titres; **D**: IgG affinity (RatioNP_2/NP_{15}). ..... : Detection threshold. IgM^b titre was measured in ELISA plates coupled with NP_2-BSA. **B**: IgG was measured in ELISA plates coupled with high hapten density (NP_{15}-BSA). **C**: was measured in ELISA plates coupled with low hapten density (NP_2-BSA). ↓: Samples with undetectable NP_2IgG, so NP_2/NP_{15} ratio is lower than the indicated value. n.s.: non-significance. $p<0.05$ between int.low and int. high avidity antibody group, according to the Wilcoxon Mann-Whitney U test. This is one of two independet experiments. LoMAv: intermediate low avidity (NP2.315), HiMAv: intermediate high avidity (NP1.298). IC: Immune complex

The size of NP-specific GC developed similar between the two groups (Fig 3.12A). Plasma cell numbers were determined in both responses. The number of NP-specific plasma cells peaks on d5. After that plasma cell numbers fall until d7 because plasmablasts come out of cell cycle, and disappear due to apoptosis or emigration (Sze-DM 2000) (Fig 3.12B). Extrafollicular plasma cell expansion is not significantly changed by variations in avidity of antibody in IC ($p>0.05$) (Fig 3.12 B). This indicates that the higher amount of high affinity IgG seen (Fig 3.11C) is not produced by variations of the extrafollicular response, but induced by effects of HiMAv IC in GCs. Taken together, these results suggest that higher avidity antibody in IC on FDCs may increase the selection threshold for centrocytes, enhancing affinity maturation during GC B cell development. Therefore, the avidity of the antibody immobilized on the FDCs seems to be important in determining the selection threshold in the GC. However, the trend to more NP^+ plasma cells in the red pulp in the group receiving HiMAv IC, may suggest that the antibody in IC may have minimal effects on extrafollicular plasma cell differentiation and proliferation as well.

In a further experiment LoAv antibody (clone82) could be used, compared to HiMAv antibody (NP1.298). Low avidity IgM^a rapidly moved into the FDC areas (Fig 3.5 A-C), and but was not detectable in GCs d5 after immunization (Fig 3.6B), while the higher avidity antibody was detectable in GCs when injecting NP1.298 (Fig 3.9B). Clone82 has a very high dissociation rate. It contains the somatic hypermutation as QM mice with an unmutated non-affinity matured antibody from BALB/c (Cascalho M 1996). This antibody should have a similar avidity to the natural antibodies cross-reacting with NP in non-immunized C57BL/6 mice, providing that the natural endogenous IgH^b antibodies of C57BL/6 can compete for antigen binding. Around day3 the first extrafollicular non-mutated plasmablasts have just formed and start to produce non-mutated low avidity antibody (Sze-DM 2000). This antibody may be able to compete with clone 82, but not

with the higher avidity clones. This would explain why clone82 started to disappear from the FDC area so early.

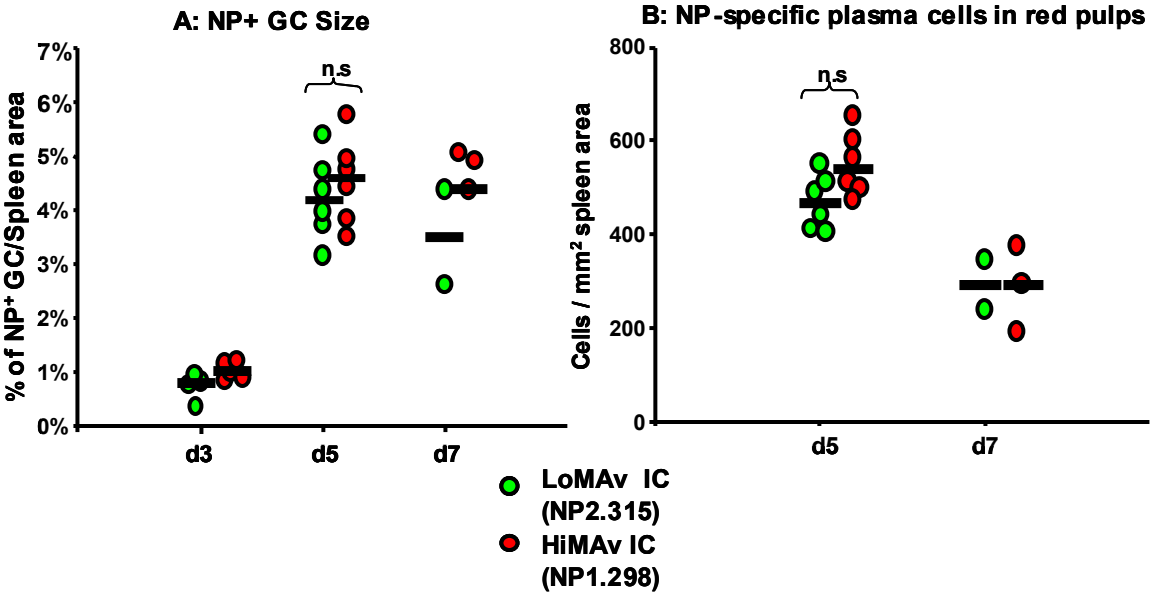

Figure 3.12: Effect of antibody avidity on GC size and plasma cell in red pulps. Germinal centre size and splenic plasma cells develop normally after immunization of LoMAv or HiMAv immune complex. **A**: the percentage of NP specific GC per spleen section. **B**: the number of NP-specific plasma cells in red pulps. Number of cells is expressed as the number per mm^2 of spleen area. Each point shows data from one mouse. n.s.: non-significance. This is one of two individual experiments. LoMAv: intermediate low avidity (NP2.315), HiMAv: intermediate high avidity (NP1.298).

3.2.3.2 High avidity antibody

To test the effects of antibody that have considerably higher avidity than the typical low affinity non-mature B cells, we injected IC prepared with HiAv antibody (NP1.197) and compared the response to IC prepared with LoMAv (NP2.315) (Fig 3.2). The same immunization protocol was used as before (Fig 3.8). Mice, primed with CGG 5 wk earlier, were *i.v.* immunized with 20μg of IC of NP-CGG plus anti-NP IgM^a of LoMAv or HiAv. The HiAv IgM^a antibody was developed from B1-8^{high} mice.

Interestingly, mice that received the HiAv IC developed significantly smaller GCs at d5

and d7 when compared to the group that received LoMAv or soluble antigen without antibody ($p<0.05$) (Fig 3.13E). The group that just received LoMAv IC developed slightly larger GCs than the group that received soluble antigen. This may be due to an adjuvant effect through costimulatory signals such as CD21 (Boackle SA 1998; Boackle SA 1998). As in the experiments described above, LoMAv IgM^a was just detectable five days after immunization with LoMAv IC (Fig3.13 A). If mice received the HiAv IC, IgM^a staining was easily observed on the FDC network in the LZ (Fig3.13 B). Binding of NP-sheep was used to detect the presence of NP-specific IC and NP-specific GC B cells. This showed that most GC B cells in the group that received LoMAv IC were binding NP-antigen (Fig 3.13 C), while in the group that received HiAv IC most of the GC B cells did not appear NP-specific, with individual GC B cells appearing NP-specific (Fig. 3.13 D). Whether this means, that most GC B cells are specific for other epitopes, e.g. the carrier protein, remains to be investigated. At the peak of the extrafollicular response on d5, antigen-specific plasma cell number in extrafollicular foci appeared significantly less in the HiAv group compared to the groups receiving LoMAv antibody or no antibody ($p<0.001$) (Fig 3.13 F). At d7 the number of plasma cells was reduced compared to d5 in all groups, with the group receiving HiAv antibody still significantly lower than the other two groups (Fig3.13F). We conclude that immunization with LoMAv IC would produce lower selection threshold than HiAv IC, leaving more GC cells alive, and so appearance of slightly bigger GCs and the generation of more plasma cells.

HiAv IC induced significantly lower numbers of plasma cells at d5 and 7 after immunization compared to LoMAv IC or free antigen. This may be due to the higher selection threshold introduced for centrocytes. Most centrocytes with lower affinity BCRs could not compete to gain access to antigen in ICs, resulting in increased apoptosis and much smaller GC output, leading to fewer plasma cells.

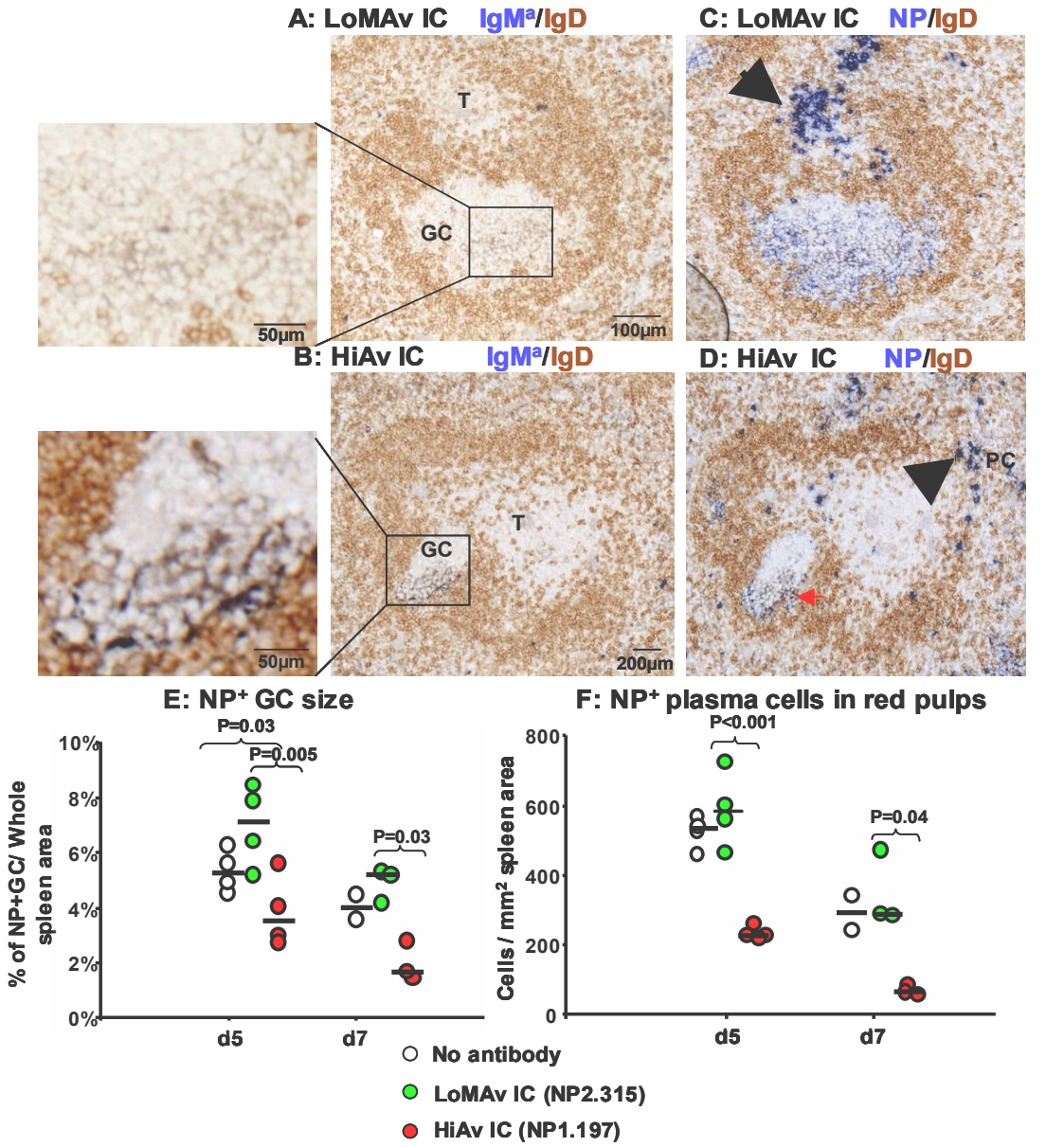

Figure 3.13: Effect of antibody affinity on GC size and plasma cell output in red pulps

CGG primed mice were injected with NP-CGG plus anti-NP IgMa with LoMAv (NP2.315) or HiAv (NP1.197). 5 days after injection LoMAv IgMa almost disappeared (**A**), while HiAv IgMa-IC is still easily detectable in GC (**B**). However, mice which received HiAv IgMa-IC developed smaller GC at day5 (**C.D**). Much fewer plasma cells produced in the extrafollicular foci (**D**). **(E)**:the percentage of NP+ GC per spleen area. **(F)**: NP-specific plasma cells in red pulps. Number of cells is expressed as the number per mm^2 spleen area. $p<0.05$ between LoMAv and HiAv, according to the Wilcoxon Mann-Whitney U test. *: $p=0.03$ between soluble Ag only and HiAv antibody group according to the Wilcoxon Mann-Whitney U test. This is one of three independet experiments. LoMAv: intermediate low avidity (NP2.315), HiAv: high avidity (NP1.197).

To study the effects of IC on selection pressure inside the GC, apoptotic cells in GCs were detected by staining for active caspase3. This showed that statistically more caspase3^{+} cells were present when mice were immunized with HiAv IC compared to mice that received LoMAv IC or soluble antigen (p=0.03, Fig 3.14A). Mice receiving LoMAv IC just had slightly more apoptotic cells in GCs compared to the soluble antigen group. At the same time GCs produced more output, reflected by a greater number of IRF4^{+} plasmablasts on the GC-T zone interface (p=0.001 Fig 3.14 B). The results prove that LoMAv IC could introduce a lower selection threshold for centrocytes, resulting in greater GC B cell survival, leading to enhanced GC output, HiAv IC in comparison supplies a strict selection threshold for centrocytes, and leading to apoptosis of more low affinity B cells in GCs, with fewer successfully selected plasmablasts.

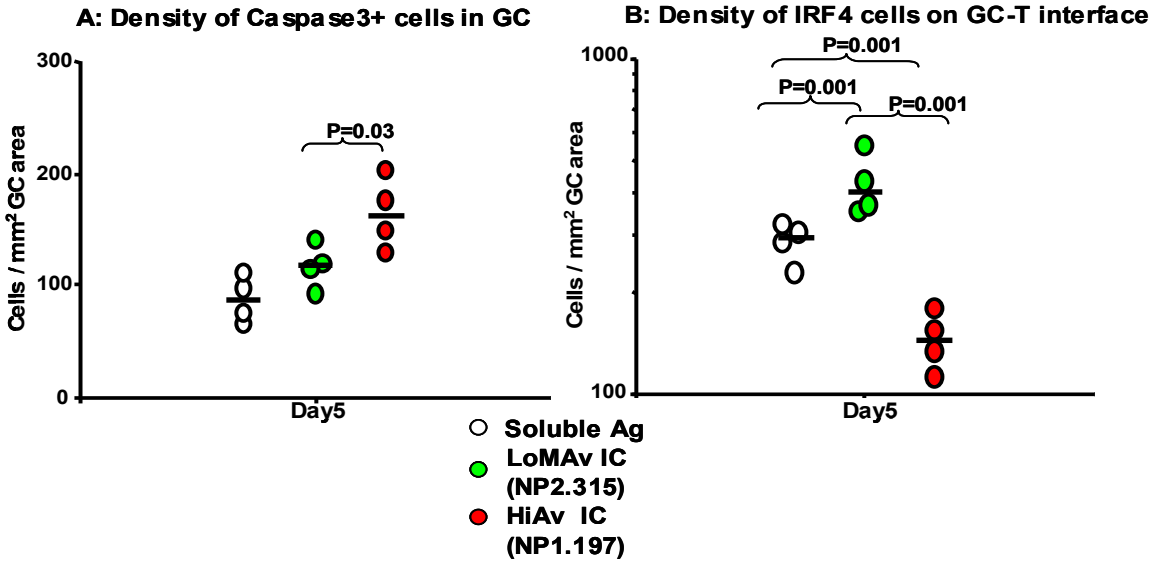

Figure 3.14: Effects of antibody avidity for apoptosis in GC and GC output
The measurement of apoptotic cells in GCs and GC outputs was used to detect selection in GC after immunisation with LoMAv or HiAv IC and only soluble antigen. (**A**) Density of Caspase3+ apoptotic cells in GC area. (**B**) Number of IRF4^{+} plasmablasts on the GC-T zone interface. Each point represents one mouse. P=0.03 between LoMAv and HiAv, according to student T test. P=0.001 between LoMAv and HiAv, between no antibody and LoMAv, or between no antibody and HiAv, according to the Wilcoxon Mann-Whitney U test. This is one of two individual experiments. LoMAv: intermediate low avidity (NP2.315), HiAv: high avidity (NP1.197). Ag: Antigen, IC: Immune complex.

To study the quality of output from GCs serum antibody measurements were done by ELISA. This showed that as in earlier experiments the production of endogenous IgM^b peaked on d5, and was similar in all groups, whether they received LoMAv, HiAv IC, or only soluble antigen (Fig 3.15A). IgG levels increased further in all groups until d7 (Fig 3.15B), and IgG affinity increased until d7 (Fig3.15 C D). Interestingly, mice that received HiAv IC had slightly lower total IgG levels, however, the affinity was significantly higher than in mice that received LoMAv IC ($p=0.02$) (Fig3.15D) or just soluble antigen ($p=0.001$).

As discussed above, HiAv IC seems to induce a stringent selection. This leads to smaller GCs, greater apoptosis and fewer plasma cells resulting in lower antibody titres, but increased antibody affinity. This effect is only noticed at the early stage day5, and disappears at day7. This effect is most prominent at early stages and is the same as the effect of HiMAv antibody for antibody response in serum shown as before (Fig3.11 C D). Mathematical modelling experiments by Michael Meyer-Hermann (unpublished) found similar effects with high avidity antibody injection decreasing GC size and GC output, while improving the affinity of the few output cells that are still produced (See section 3.3.4 Discussion).

3.2.4 The impact of soluble extraneous antibody for B cell responses

The experiments described above indicate that extraneous antibody is deposited on FDCs, and may affect GC B cell selection threshold depending on the form of antigen-antibody IC at the time of immunization. Our competition model predicts that the GC selection threshold is determined by the antibody produced by plasma cells, which have developed in extrafollicular foci during the ongoing immune response. If this model is correct, then systemic antibody should freely diffuse back into the established GC. To test this, we injected soluble extraneous antibody injected during ongoing GC responses.

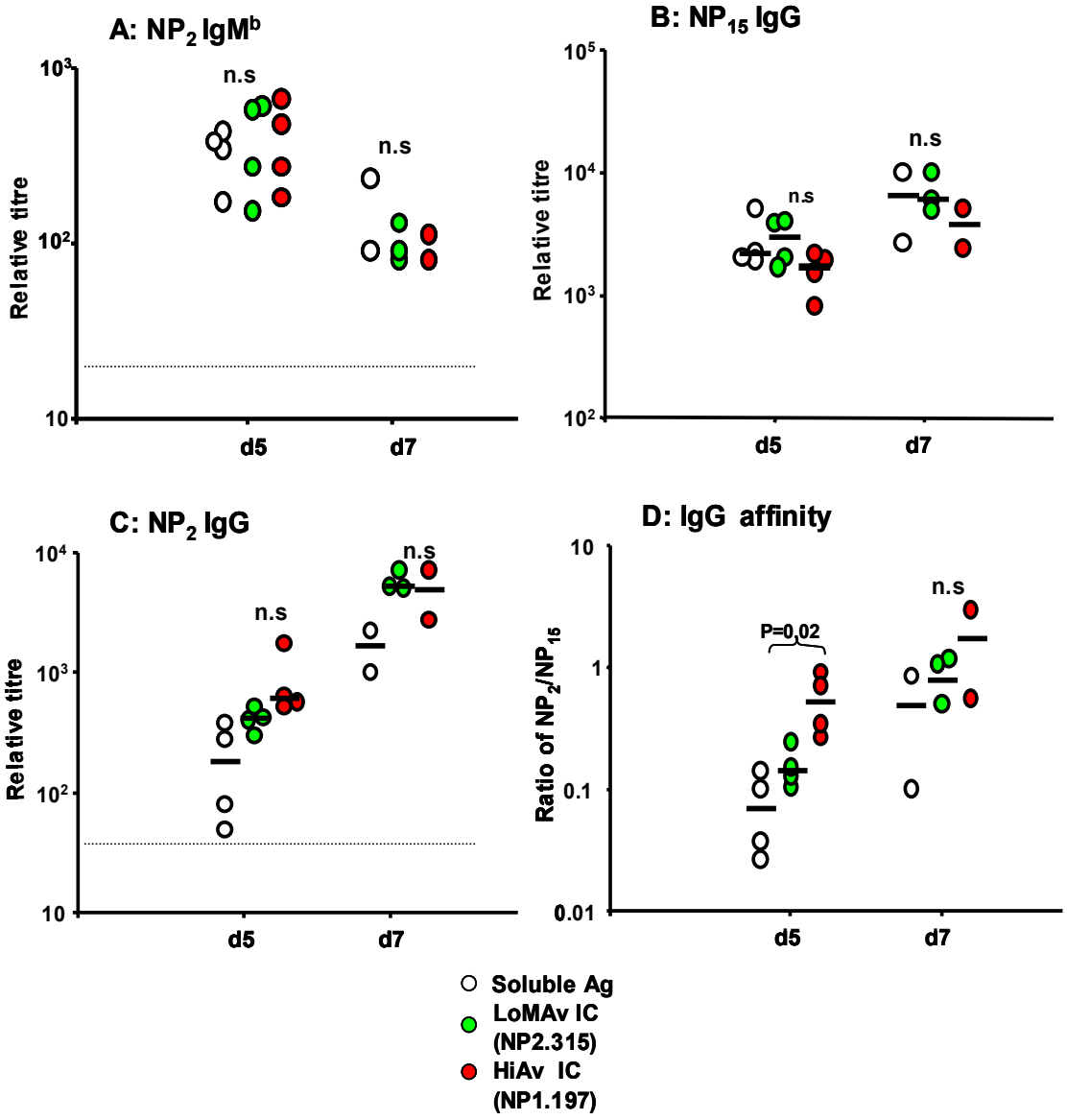

Figure 3.15: IgM^b titre, IgG titres and affinity in serum

The figure shows the relative IgM^b and IgG antibody titre at the following time course: d5 and 7 after injection with LoMAv or HiAv IgM^a-IC at the CGG primed mice. Each point represents one mouse. **A**: (NP_2) IgM^b titres; **B**: (NP_{15}) IgG titres; **C**: (NP_2) IgG titres; **D**: IgG affinity (Ratio of NP_2/NP_{15}). ..... : Detection threshold. IgM^b titre was measured in ELISA plates coupled with NP_2-BSA. **B**: IgG titre was measured in ELISA plates coupled with high hapten density (NP_{15}-BSA). **C**: IgG was measured in ELISA plates coupled with low hapten density (NP_2-BSA). n.s.: non-significance. P=0.02 between LoMAv and HiAv antibody group, according to the Wilcoxon Mann-Whitney U test. This is one of three individual experiments.LoMAv: intermediate low avidity (NP2.315), HiAv: high avidity (NP1.197). Ag: antigen, IC: Immune complex.

We used alum-precipitated NP-CGG to induce GC in C57BL6 mice at day10 of the primary response and soluble NP-CGG at day 4 after the carrier-priming. In the first experiment 20μg of soluble LoAv IgM^a (Clone82) was given via *i.v.*. Interestingly no allotype IgM^a was detectable on FDCs in GCs at any time point 2hr, 1, 2, and 4d after injection. Possibly the dose of antibody used was too low. We injected mice with 80μg, 200μg and 500μg of LoMAv IgM^a (NP2.315) at day10 after primary response with NP-CGG. IgM^a still was not detectable on FDCs. Clone82 and NP2.315 are low avidity antibodies, as demonstrated by BiaCore analysis (Fig 3.2 E). From this we concluded that 10 days after the primary response or 4 days after 2nd injection in the carrier-primed mcie, GC development may be too advanced, with GC derived plasma cells in the red pulp already producing considerable amounts of high affinity antibodies. According to the antibody competition model this would appear on FDCs, and prevent extraneous antibody from entering the GC.

In the next series of experiments, higher avidity soluble antibodies were injected at very early stage of GC development. At day3 and day4 after carrier-primed response to NP-CGG, 90μg of LoMAv (NP2.315) or HiAv (NP1.197) antibody was given *i.v.* (Fig 3.16). Immunohistological staining was used to detect extraneous IgM^a on spleen section at day5 after immunization.

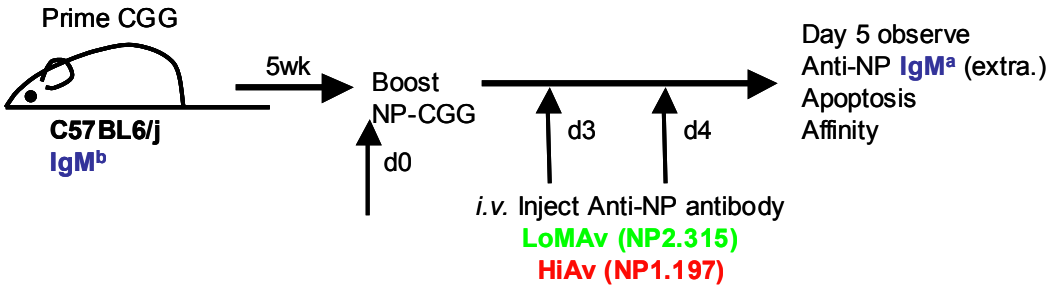

Figure 3.16: Soluble int. low or high avidity antibody was introduced at d3 and d4 after NP-CGG immunization in carried-primed mice, to further detect the impact of soluble antibody for B cell selection in GC.

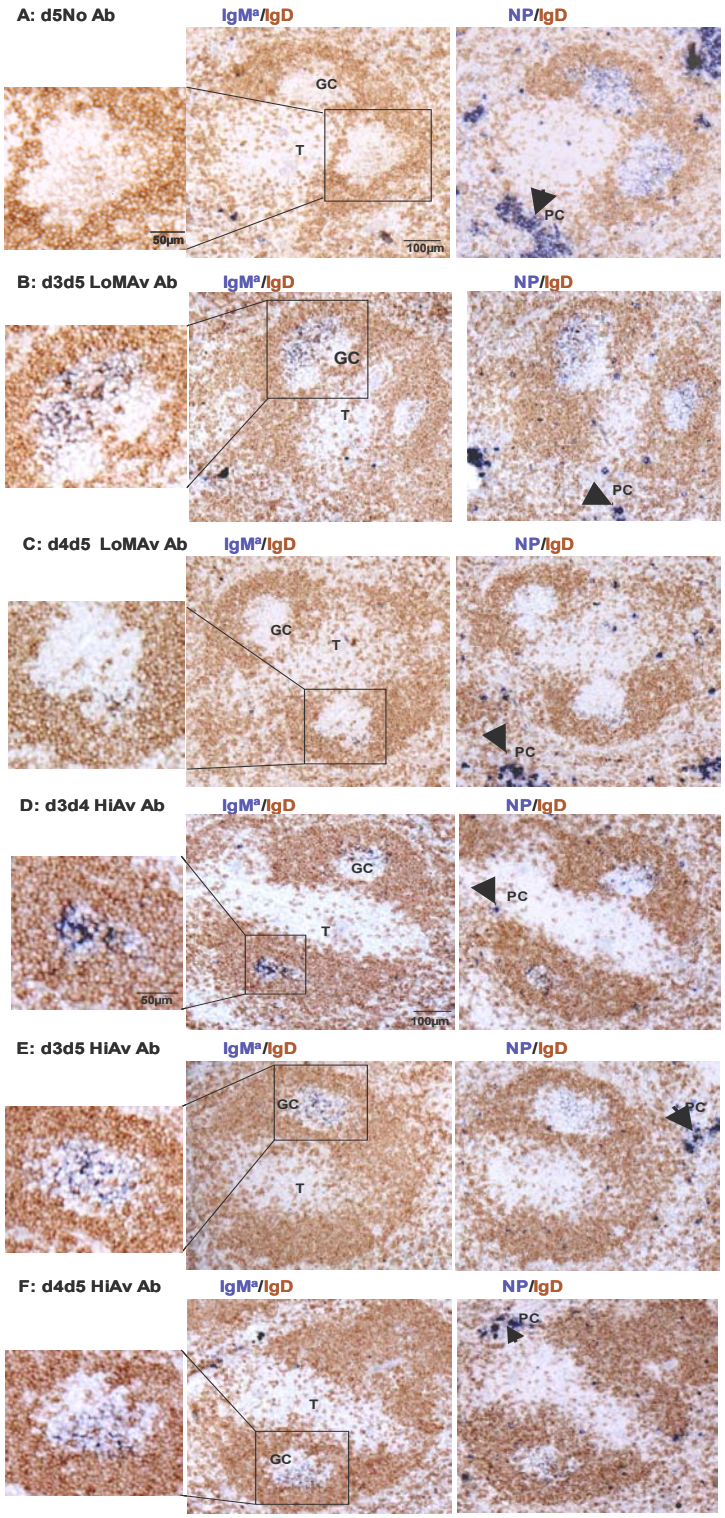

Figure 3.17: Soluble LoMAv/HiAv antibody can move into the early developed GC

CGG primed mice were injected with NP-CGG, at d5 the spleen tissue were collected as control (**A**). At d3 and 4, *i.v.* immunized with soluble LoMAv Ab, and at 5, extraneous IgM^a was detected on section (**B** and **C**) . If at d3, mice were received HiAv Ab, d 4 extraneous IgM^a was detected on section (**D**). If at d3 and 4, mice were gave HiAv Ab, at d5 extraneous IgM^a was detected on section (**E** and **F**). At the same time. GC and plasma cells were assessed by staining with NP. LoMAv: intermediate low avidity (NP2.215). HiAv: high avidity (NP1.197). GC: Germinal centre, PC: plasma cell.

In contrast to the LoAv antibody experiment described above, LoMAv IgM^a antibody could be seen very clearly in GCs on all sections at day5 after immunization if antibody was injected at day3. However, if LoMAv antibody was injected one day later, there was less present in GCs on day 5 (Fig 3.17 B.C). This may be explained by affinity of endogenous antibody already competing with deposition of this low avidity antibody by day 4. When HiAv antibody (NP1.197) was used for injection 3 days after NP-CGG immunization, it was found deposited on FDCs in high densities within 24hr (Fig. 3.17 D). HiAv antibody was still detectable on the FDC network at high amounts two days after injection (Fig. 3.17 E). Different from LoMAv, HiAv was injected 4 days after NP-CGG was still easily detected on the FDC network 1 day later (Fig. 3.17F). This confirms that an affinity dependent process seems to prevent LoMAv antibody from entering the GC on day 4, indicating that there may be competition with higher avidity endogenous antibodies that have appeared at this stage.

The size of NP-specific GC on day 5 was significantly smaller in the carrier-primed mice receiving soluble LoMAv or HiAv antibody at day3 or day4 compared to mice that only received soluble antigen ($p=0.01$ Fig 3.18A). No significant difference was detected between LoMAv and HiAv groups, although there was a trend to smaller GC in the HiAv group. Accordingly, both affinities of antibody induced a strong rise in the frequency of apoptotic cells in GCs, as measured by staining for active caspase3, indicating that these antibodies have an affinity-dependent effect on the selection threshold (Fig 3.19A). HiAv antibody induces significantly more apoptosis than lower avidity antibody (Fig. 3.19A red points). LoMAv induces less apoptosis than HiAv antibody, but significantly increases apoptosis compared to the GC, which did not receive extraneous antibody (Fig. 3.19A green points). Interestingly, the effect of LoMAv was dependent on the time when this antibody was injected. Injecting LoMAv antibody on day 3 resulted in significantly more

apoptosis detected on day 5, than when it was given on day 4, indicating that this LoMAv antibody is less able to compete with the endogenous antibody, when given on day 4.

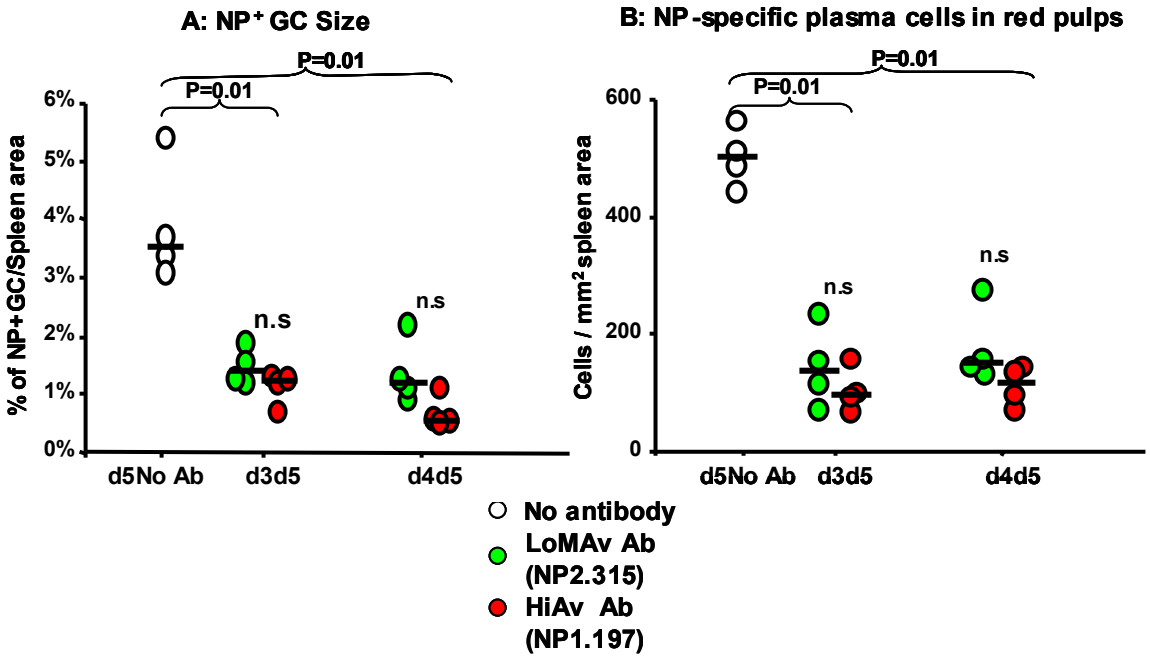

Figure 3.18: Effect of antibody avidity on GC size and plasma cell output in the red pulps

CGG primed mice were injected with NP-CGG, at day3 and 4, *i.v.* immunized with soluble LoMAv or HiAv antibody, and at d5, samples were collected called d3d5, and d4d5 respectively. Another group of mice did not get soluble antibody as control labeled as d5No Ab. **(A)**: the percentage of NP^+ GC per spleen section area. **(B)**: NP-specific plasma cells in red pulps. Number of cells is expressed as the number per mm^2 spleen area. P=0.01 between no antibody injection and antibody immunisation group, according to the Wilcoxon Mann-Whitney U test. Each point represents one mouse. This is one of two individual experiments. LoMAv: intermediate low avidity (NP2.315). HiAv: high avidity (NP1.197).

Appearance of IRF4 positive cells at the GC–T zone interface, a marker for early plasmablast output from the GC, showed significantly fewer plasmablasts after mice received soluble extraneous antibodies than in the absence of extraneous antibody

(p<0.05, Fig 3.19 B), confirming that the selection threshold in the GC is higher, resulting in less output. Injection of antibody also resulted in significantly fewer plasma cells in the red pulp (p=0.01 Fig 3.18 B). No significant difference was detected between the different avidity antibodies.

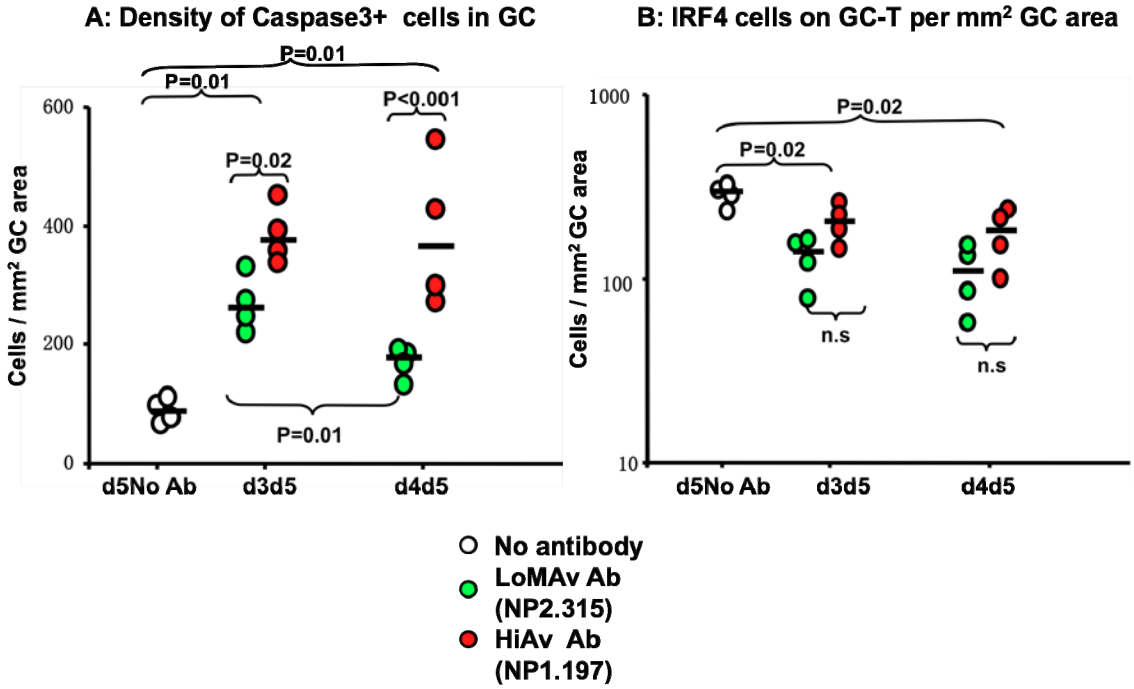

Figure 3.19: Injection of soluble LoMAv/HiAv Ab induces more caspase3$^+$ cells in GC, and decreases GC output

CGG primed mice were injected with NP-CGG, at day3 and 4, *i.v.* immunized with soluble LoMAv or HiAv antibody, and at d5, samples were collected called d3d5, and d4d5 respectively. The apoptotic cells in GC and GC outputs were assessed after immunisation with int. low/high avidity soluble antibody. (**A**) Density of Caspase3+ apoptotic cells in GCs. (**B**) Number of IRF4$^+$ plasmablasts on the GC-T zone border. Number of cells is expressed as the number per mm^2 GC area. p<0.05 between int. low and high avidity group, between no antibody injection and antibody immunisation group according to the Wilcoxon Mann-Whitney U test. n.s.: non-significance. Each point represents one mouse. This is one of two individual experiments. LoMAv: intermediate low avidity (NP2.315). HiAv: high avidity (NP1.197)

ELISA was used to detect antibody production in blood. This showed production of total IgG and high affinity IgG 5d after immunization with NP-CGG (Fig 3.20A, B). Interestingly, High avidity antibody induced significantly more higher affinity IgG production when injected at day3 and 4 after NP-CGG injection in the CGG-primed mice ($p<0.05$, Fig 3.20 C), even though no significant difference was detected in the total IgG and in the high affinity IgG production between groups receiving antibody on day3/4 and no antibody injection group.

LoMAv antibody has a smaller effect for the B cell reaction in GC. This is similar to results shown earlier (section 3.2.4.2, Fig 3.14), when mice were immunized with LoMAv IC. This is compatible with the idea that soluble HiAv antibody moving into GCs would increase the selection threshold leading to stringent selection which allows only higher affinity B cells to be selected. This leads to higher affinity antibody production. The results from injection with soluble antibodies are in line with the results of the experiments with IC immunization, and are compatible with the competition model that: 1) antibody in IC in GCs may be replaced by antibody formed later in the response, which then induces the antibody affinity on FDCs to increase during the process of the immune response, 2) centrocytes compete with antibodies on FDCs for antigen binding, 3) the affinity of antibody immobilised on FDCs is important to determine the selection threshold of GC B cells.

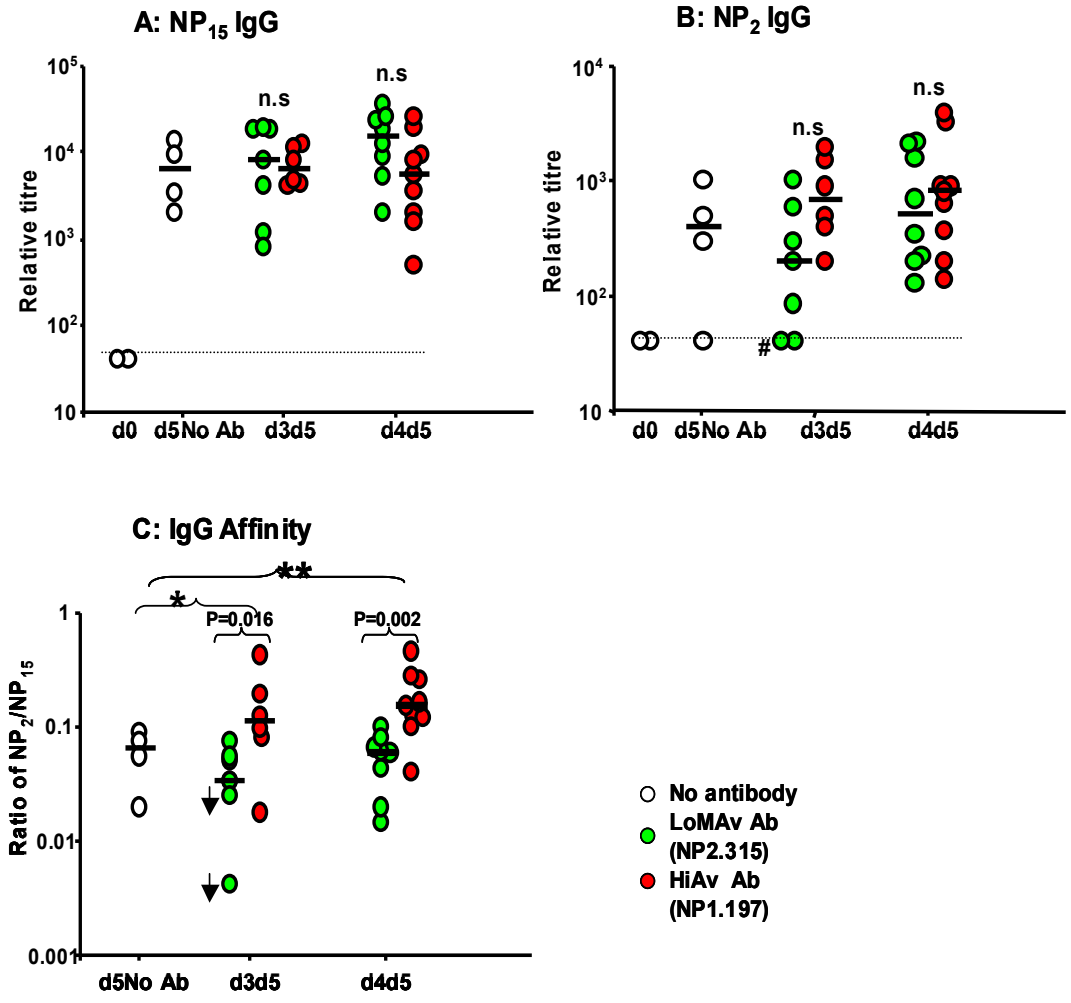

Figure 3.20: IgG Ab titres and affinity

The figure shows the relative IgG antibody titre and affinity at the different time course. CGG primed mice were injected with NP-CGG, at d3 and 4, *i.v.* immunised with soluble LoMAv or HiAv antibody, and at d5, antibody in sera were assessed called d3d5, and d4d5 respectively. Another group of mice did not get soluble antibody as control labeled as d5 No Ab. **A**: (NP_{15}) IgG titres; **B**: (NP_2) IgG titres; **C**: IgG affinity (Ratio of NP_2/NP_{15})). ..... : Detection threshold. n.s.: non-significance. *: p=0.04, **: p=0. 01 between non antibody injection and HiAv injection group, $p<0.05$ between LoMAv and HiAv, according to the Wilcoxon Mann-Whitney U test. #: not detectable with NP_2BSA, so value is as plotted or lower. ↓ : the same two samples shown on Fig B, so the value actually is lower than the plotted. Each point represents one mouse. This is the merge of two individual experiments. LoMAv: intermediate low avidity (NP2.315). HiAv: high avidity (NP1.197)

3.2.6 The effect of antibody on the extrafollicular response

All the experiments described above assume that antibody has effects solely on selection events in GCs. B cells initially get activated outside the GC, and there may be effects on competition between antibody and B cell at the pre-GC stage. To investigate whether antibody avidity of IC could affect the extrafollicular response, we studied the response of QMxC57BL/6 mice to the TI antigen (NP-Ficoll), and IC in the form of NP-Ficoll covered in either IgD (low avidity heavy chain, similar to clone82 but bivalent. Fig 3.2E) or IgM antibody (clone82) (for the preparation see section 2.2). Spleens were harvested at day3 after immunization, which is the timepoint when B blasts differentiate into plasmablasts in the red pulp, and when the first GC appear in follicles (Vinuesa CG 2000). QM mice produce a strong extrafollicular response to NP-Ficoll and develop GCs, but do not show hypermutation or affinity maturation ((Vinuesa CG 2000; Toellner KM 2002). Thus, at day 3 after immunization all B blasts and plasmablasts have developed outside GCs, and there is no extra competition from affinity matured endogenous antibody.

GC developed after mice were immunized with NP-Ficoll alone, or both kinds of ICs (Fig 3.21A), but in terms of GC size, no significant difference was found in all groups after injection ($p > 0.05$), and this is similar to the results of the effect of LoMAv IC for GC (Fig 3.13 E). High population of NP^+ plasma cells were produced in red pulps after immunization with antigen only or IgD-ICs/IgM-ICs (Fig 3.21B). There were only slightly fewer NP-specific B cells in the extrafollicular foci after receiving IgM-IC ($p>0.05$). This result shows that antibody avidity has only a minimal role for the development of B cells in the extrafollicular response. In addition, two different avidity ICs of IgM plus NP-Ficoll were injected QMxC57BL/6 mice. No significant difference in GC size, or in the production of plasma cells and serum antibody were detected in the between groups (L. George MSc Thesis, UoB). Taken together, these results show that most of the effects seen on GC size, plasma cell numbers and serum antibody affinities in

the experiments described above are mainly due to effects inside the GC, and minimum effects on early B cell activation in the extrafollicular pathway. The density change of antigen-specific plasma cells in red pulps induced by different avidity ICs originates from the direct effect of IC for the GC development.

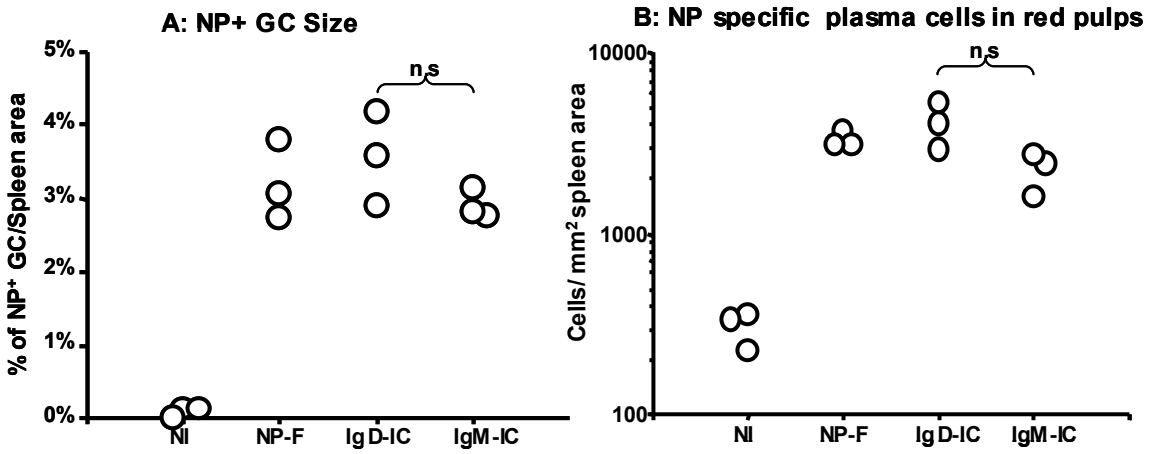

Figure 3.21: Little effect of antibody of different avidity on the extrafollicular response

QMxC57BL/6 mice were immunized with the TI antigen NP-Ficoll, NP-Ficoll plus anti-NP IgD or IgM immune complex, at day3, GC size and NP^+ cells were quantified on spleen sections. **A**: The percentage of NP^+ GC size of whole spleen section, **B**: NP binding plasma cells in the red pulp. Number of cells was accessed per mm^2 spleen section. NI: Non-immunized. n.s: non-significance. IC: Immune complex. Each point represents one animal.

3.3 Discussion

B cells, activated by antigen and T cells, move into follicles and establish the GCs which are an important site for the generation and selection of B cells bearing high-affinity antibodies (MacLennan 1994). In the classical model of GC function, B cells in the DZ undergo rapid rounds of proliferation and somatic hypermutation of their Ig V region genes, followed by exit from the cell cycle and movement into the LZ, where they undergo selection based on the affinity of their surface antibody for antigen (MacLennan 1994; Manser 2004; Tarlinton 2006) Selection process is thought to involve competition between GC B cells for antigen capture in the form of immune complex displayed on the processes of FDCs (Cyster JG 2000; Cyster JG 2000; Kosco-Vilbois 2003). Recent real time imaging studies also support this idea (Schwickert TA 2007; Allen CD 2007a; Hauser AE 2007b). When antibody from the primary immunization is present, antigen-antibody immune complexes form and are trapped by FDCs (Tew JG 1997). Deposition of immune complexes on FDCs is strongly dependent on complement and Fc receptors (Allen CD 2008). It is widely accepted that immune complex on FDCs play a major role in B cell maturation (Song H 1999; Aydar Y 2005) and differentiation (Tarlinton DM 2000; Haberman AM 2003). In addition, some studies have indicated an important feedback role of early primary antibody such as IgG1 in the regulation of the B cell repertoire and activation of somatic hypermutation in GC B cells (Nie X 1997; Song H 1999). Here we sought to determine whether antibody in immune complex on FDC is involved in B cell selection by competing with centrocytes for access to antigen in GCs.

As the affinity of the BCR for GC B cells increases with time, it should become easier and easier for B cells to compete with antibody in IC on FDCs. A mechanism based on increase in the stringency of selection through the replacement of the initial antibody by external antibody would overcome this problem. This external antibody is produced by

GC derived plasma cells, and would warrant efficient selection while higher affinity B cell clones develop. A theoretical study that was done in parallel to the experiments described here showed that a dynamic selection threshold dependent on antibody affinity in IC, is a more efficient mechanism to achieve selection of high-affinity clones (Adam Reynolds 2010, thesis UoB, submitted). This study led us to investigate whether the affinity of antibody immobilized on FDCs is an important factor for germinal centre B cell selection.

3.3.1 Appearance of immune complex in follicles

The first experiments described here tested the speed of transportation of IC into follicles. To help visualize the migration of IC containing extraneous antibody by immunohistology, different allotype (a/b) of the same isotype antibody was used. Immunohistological staining shows that extraneous IgM^a-IC is quickly transported towards the follicles from the marginal zone within 2h after *i.v.* injection. Within a day all IgM^a-IC localizes on FDC in the light zone.

The marginal zone is considered to screen the systemic circulation for antigens and pathogens (Mebius RE 2005). Earlier studies of the capture and deposition of systemic immune complex in the spleen suggested that some lymphocytes migrate from the marginal zone to the follicle (Brown JC 1970; Veerman AJ 1975). MZ B cells were reported to have a role in the transport of IC (Ferguson AR 2004). The light zone in the GC is proximal to the marginal sinus where blood-borne antigens enter the tissue. Antigen enters the spleen via blood draining the marginal sinus, MZ B cells engage TD or TI antigen through their BCR, which is further crosslinked by CR2, upon which they are then induced to leave the MZ and migrate to the outer T zone at the first stage in a splenic antibody response (Liu YJ 1988; Vinuesa CG 2001; MacLennan 2008). When IgM-IC

was directly injected *i.v.*, IgM-IC localized firstly at the MZ where IgM-IC bound MZ B cells in a complement and complement receptor (CR1/2) dependent process. Then MZ B cells transported the IgM-IC into the follicle for deposition onto FDCs (Ferguson AR 2004). In addition, Cinamon (2008) presents that MZ B cells are not confined to the MZ but continuously shuttle between the MZ and follicular areas by up-regulating the expression of the chemokine receptor CXCR5 (migration to follicle), or sphingosine1-phosphate receptors $S1P_1$ and $S1P_3$ (back to MZ). This continuous shuttle provides an efficient mechanism of systemic antigen capture and delivery to FDCs (Cinamon G 2008). The same is observed in lymph nodes, where the light zone is positioned close to the subcapsular sinus, which receives the afferent lymphatic drainage from the skin, mucosa, or viscera. Naïve B cells have been shown to frequently visit the follicles, or an ongoing GC reaction (Schwickert TA 2007). Follicular B cells may play a transport role in lymph nodes for larger antigens such as IC or viruses in lymph nodes (Carrasco YR 2007; Junt T 2007; Phan TG 2007). Phan (2007) indicated that follicular B cells captured ICs through a complement receptor-dependent mechanism from subcapsular macrophage processes and then carried complexes through the follicle, and/or migrated to the T-B zone boundary for delivery to FDCs.

Antigen in the form of IC could last longer (Tew JG 1978), and was reported to be typically more potent than free antigen in promoting antibody responses (Heyman 2000). Previous studies had suggested that antigen retained in lymphoid follicles remains active for prolonged periods, and could maintain and regulate serum antibody levels for months or years after immunization (Nossal G. 1968; Tew JG 1973; Tew JG 1978; Tew JG 1984).

The kinetics of the appearance of IgM-IC in the MZ, followed by the follicle, described here during a carrier-primed response, was similar to antigen trapping in the subcapsular

sinus and then transport into the follicles in lymph nodes, when antigen was injected into animals primed with the same antigen (Szakal AK 1989), or phycoerythrin (PE) was immunized into mice primed with polyclonal antibodies specific for PE (Phan TG 2007). Immune complex rapidly formed after injection, or directly injected IC was captured by antigen-transporting cells and subsequently accumulated in the FDC area.

Recent studies using two-photon microscopy reported that small molecular soluble antigens could gain access to cells in the follicles of lymph nodes through direct diffusion through the subcaspular sinus pores (Pape KA 2007) or through follicular conduits (Roozendaal R 2009). In our experiments, soluble antibody (particularly high avidity) was detected in the FDC area at day1 after immunization during an ongoing GC reaction. Therefore in the spleen, the antibody may also rapidly diffuse across the marginal zone into the GC, and then be retained on FDCs. Whether soluble antibody enters the follicle with increase kinetics has not been tested in this project. However, as a small molecule it should be faster. Therefore, internally produced higher affinity soluble antibodies are likely to gain access to germinal centres and then provide feedback for the GC B cells selection.

3.3.2. The links between FDC, antibodies and antigen

A range of i*n vivo* investigations provide clear evidence that complement receptor CR1/CR2 (Nielsen 2000), FcγRIIB (CD32), and FcεRII (CD23) (Qin D 2000; Ravetch JV 2008; Niederer HA 2009) are involved in antigen trapping, particularly the deposition and retention of immune complex on FDCs. IgM is a very efficient activator of complement (e.g.C1q) (Getahun A 2006). The complement C3 is cleaved into fragments, first C3b, and then C3dg and C3d. Bond C3 acts as a costimulator to B cells via CD21 and CD19 (Fearon DT 2000). FDC binds C3b via the complement receptor CR1 (CD35)

and C3d via CR2 (CD21) (Tew JG 2001; Carroll 2004). CR1 and CR2 expressed on FDC are important for presentation of antigen to B cells and the induction of immunologic memory (Toapanta FR 2006; Speth C 2008). On phagocytes, CR1 cooperates with FcγRIIB to bind and ingest foreign material (Ravetch JV 2008). CR2 is thought to cooperate with FcεRII, the low affinity receptor for IgE, to regulate the production of IgE (Aubry JP 1992). IgG immune complexes bind the low affinity FcγRII (10×10^6 M^{-1}) on FDC (Ravetch JV 2008; Niederer HA 2009). Possibly, IgM-IC deposition on FDC is mainly dependent on CR1 and CR2. It has been proposed that this could increase the effective concentration of antigen available to stimulate germinal centre B cells (Tew JG 1997).

Fcα/μR was recently identified as a high affinity Fc receptor for IgM (~3×10^9 M^{-1}), and intermediate affinity receptor for IgA (Shibuya A 2000; Kikuno K 2007; Kubagawa H 2009). The Fcα/μR gene is localized on chromosome 1 (Shibuya A 2000; Shimizu Y 2001), close to other Fc receptor genes, including FcγRI, II, III, and IV (Daeron 1997; Nimmerjahn F 2006), the polymeric IgR, and Fc homologues (Davis RS 2001). Fcα/μR is expressed on hematopoietic cells (e.g. B cell and macrophage) and various nonhematopoietic organs including liver, kidney, small and large intestines, testis and placenta, but not on granulocytes or T cells. In the mouse spleen, Fcα/μR is expressed preferentially on FDC, MZ B cells, and follicular B cells (Kikuno K 2007; Honda S 2009). Recent work in Fcα/μR knock-out mice suggests that the receptor negatively regulates antigen retention by FDC, resulting in the suppression of GC responses. This was conluded from the presence of germinal centres in responses that develop in the absence of Fcα/μR to TI-II antigen (Honda S 2009). Fcα/μR is believed to function by trapping IgM immune complexes and presenting the intact antigen to B cells in germinal centres (Kikuno K 2007; Kubagawa H 2009; Czajkowsky AM 2010). During the early immune response, IgM is the predominant Ig isotype, therefore Fcα/μR may have more

pronounced effects than FcγRII for the presentation of immune complex on the FDC network in the early germinal centres.

Antigen opsonized by IgM and complement as immune complex may be captured on FDC by binding to CR1/CR2 and/or Fcα/μR (Fig 3.22). The binding of CR2 to C3d is complex, possibly involving bivalent interactions (Sarrias MR 2001). The association of C3d with CR2 is of electrostatic nature and therefore reversible (Morikis D 2004; Toapanta FR 2006). The affinity of this interaction is relatively low (K_D: $\sim 5 \times 10^7\ M^{-1}$) (Sarrias MR 2001). Interaction of CR1 with complement is also reversible. As mentioned above Fcα/μR binds IgM with relatively high affinity, but this is thought to be a monovalent interaction (Shibuya A 2000). This means that, apart of the interaction of C3 with antibody/antigen, all bonds between FDC and antigen are reversible. More importantly, all interactions are in the range of the avidities of the multimeric IgM antibody present in the early immune complex, making it possible that IgM is released from the immune complexes on FDCs as soon as the IgM – antigen bond is broken due to competitive binding of higher avidity endogenous antibody (Fig. 3.22).

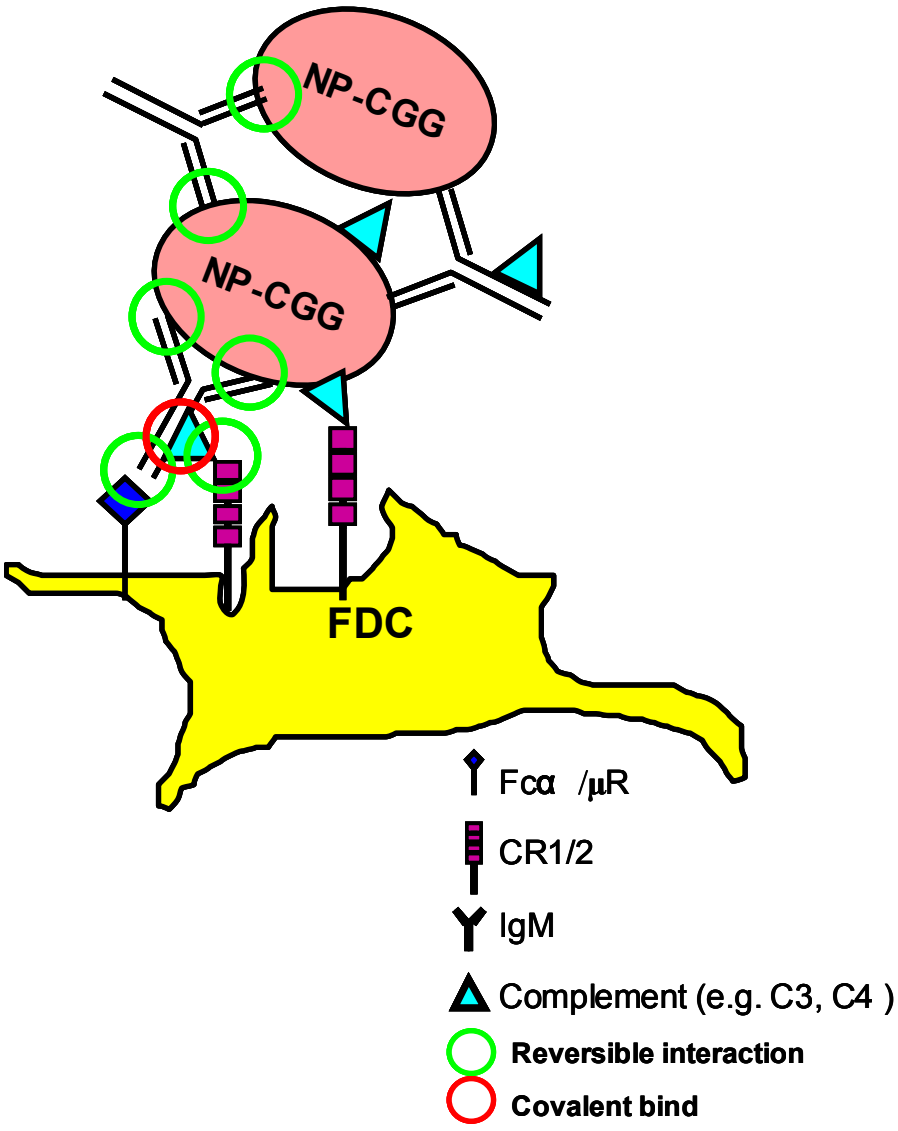

Figure 3.22: The proposed binding mechanism of immune complex on FDC
Antigens opsonized by IgM and complement as immune complex are captured on FDC by binding to CR1/2 and/or Fcα/μR. The affinity of C3d with CR2 interaction is relatively low, and reversible (green circle). Interaction of CR1 with complement is also reversible (green circle). Fcα/μR binds IgM with relatively high affinity, but this is thought to be a monovalent interaction (Green circle). Apart of the interaction of C3 with antibody/antigen, all bonds between FDC and antigen are reversible. All interactions are in the range of the avidities of the multimeric IgM antibody present in early immune complex. Possibly IgM is released from the ICs on FDC as soon as the IgM – antigen bond is broken due to competitive binding of higher avidity endogenous antibody.

3.3.3. Is antibody replacement dependent on antibody avidity?

Our hypothesis of an antibody avidity dependent selection threshold in GCs predicts that antibody inside the GC is in a dynamic equilibrium with antibody outside the GC. Antigen has been shown to be retained on FDCs for extended period of time (Mandel T 1980). Antibody in IC should be replaced by higher avidity antibody, as the GC reaction proceeds. If this process is dependent on avidity, antibodies in GCs of different avidity should have different half-lives. Testing this gave two results. At the time when the highest amount of extraneous IgM^a was seen on FDCs, there seemed to be a loss of endogenous IgM^b at the same locations. Conversely, at later stages, IgM^a-IC seen in GCs started to disappear, and was hardly detectable in GCs by day5. During the same time, high amounts of endogenous IgM^b-IC were seen on FDCs. GCs contained few antigen-specific (NP^+) B cells at day3, and by day5, large NP-specific GCs were established, while considerable numbers of plasma cells in the extrafollicular foci produced amount of NP-specific antibody. Antigen specific B cells that are activated during the first 2 days after NP-CGG immunization in CGG-primed mice, undergo extensive proliferation either in extrafollicular foci or as a part of the GC reaction (Toellner KM 1996). Antibody production from new NP^+ plasmablasts starts after day3, coinciding with the time when sporadic NP-specific cells and small antigen-specific GCs were seen. The number of NP^+ plasma cells in the red pulp peaks at day5 when most of the cells come out of cell cycle and GCs reach their peak size at around day4. Some of the NP^+ plasma cells in the red pulps at day5 were found to have mutated V region sequence and therefore appear to be early emigrants from GCs (Sze DM 2000). Here, we found high numbers of NP^+ plasma cells produced in extrafollicular foci, a fraction of these are likely to have developed from large antigen-specific GCs, where they may have acquired mutations, producing higher affinity B cell receptor that led to their positive selection. These cells would produce higher affinity antibody. It seems likely that these higher avidity IgM^b antibodies generated from new plasma cells could diffuse back to

replace the low avidity IgM^a in IC, leading to changes in the selection threshold. Even though this experiment did not directly observe the replacement process in the tissue, it seems likely that antibody retained on FDCs is dynamic and capable of being replaced during an immune response.

The LoAv antibody clone82 was shown by BiaCore to be the lowest avidity antibody used in this experiment. It has a very fast on-rate and also a very fast off-rate (dissociation rate). Avidity (and affinity) is a factor derived from association and dissociation rate, although a slow dissociation rate is considered to be the primary determinant for antibody affinity (Andersson K 1998; Batista FD 1998). This explains why clone82 quickly disappeared from IC on FDCs.

Soluble antibody injected rapidly moved into the FDC network during an ongoing GC response. This result may imply that the replacement of antibody in immune complexes is a passive transport. To provide further evidence for this, new experiment should be designed to detect high and low avidity antibodies. This could be done by marking antibodies of different affinities, e.g. FITC or biotin, and following their deposition on FDCs.

HiMAv IgM^a-IC was still easily detectable in GC at day5 after injection while LoMAv IgM^a-IC had almost disappeared. This suggests that avidity could influence replacement in IC, so there must be on-off binding of the antibody to the antigen, particularly dynamic equilibrium of antibody from outside GCs and inside. This means that antibodies are not fixed in immune complexes on FDCs, e.g. hidden behind cell membranes as iccosomes or covalently linked via complement receptors, although a proteinase dependent process could release even covalently fixed antibodies (Andersson K 1998).

3.3.4 The impact of different avidity immune complex in GC B cell selection

IC can facilitate rapid activation of lymphocytes, indeed captured and deposition by antigen-presenting cells (APCs) can induce faster and/or stronger GC development compared to immunisation with antigen alone (Kunkl A 1981; Ehrenstein MR 1998; Ferguson AR 2004; Corley RB 2005). IgM-IC is deposited on FDCs, dependent on their expression of complement receptor CR1/CR2 (CD35/CD21) and may thereby increase the effective concentration of antigen available to stimulate B cells (Tew JG 1997). Physical association between CD21with CD19 and CD81 (TAPA-1) on the surface of B cells, lowers the threshold for B cell activation (Boackle SA 1998). This may explain our finding that immunization with IgM-IC can improve the GC B cell responses. A recent study reported that IgM may have function in concentrating antigens in secondary lymphoid organs and stimulating both CD4 T cells and B cells, efficiently facilitating GC formation (Corley RB 2005).

In line with this, IgM^a-IC rapidly promoted GC formation during the carrier-primed response to NP-CGG. Furthermore, the avidity of antibody in IC could affect the selection of GC B cells. ELISA data showed that although no difference in the total amount of endogenous IgM^b production was seen in between the HiMAv and LoMAv groups, higher affinity Ig was detected at day5 after mice received higher avidity IC at the early stage of GC development. GC B cells scan antigen trapped on FDCs (Schwickert TA 2007). If avidity dependent competition for antigen access is important, HiMAv IgM^a-IC may give higher avidity GC B cells a selection advantage. These B cells may expand better compared to an environment with a low selection threshold, producing a greater plasma cell output, resulting in the early onset of high avidity antibody.

There are several possible mechanisms how B cells may compete for antigen-derived signals. GC B cells are prone to apoptosis. Isolated GC B cells will die within hours,

unless survival signals such as BCR stimulation and CD40 dependent signals are received (Liu YJ 1989). BCR mediated signals are dependent on access to antigen. Signals from BCRs without high enough affinity for antigen will just receive a small or brief signal, and will die (Tarlinton DM 2000). Selection may be due to direct competition between B cells due to limited access to the antigen because of spatial restrictions (Meyer-Hermann ME 2006), or affinity dependent access to antigen. Competition for space is not affinity dependent, therefore a further selection process on T cells is necessary (Meyer-Hermann ME 2006; Allen CD 2007a; Allen CD 2007b). A single GC T cell is suggested to only form a stable interaction with GC B cells that present the highest number of antigen peptide-MHC class II complexes compared with neighbouring cells (Allen CD 2007b). Therefore high affinity GC B cells would gain an advantage in obtaining T cell help. Direct competition between B cells may also be dependent on the affinity of the BCR (Andersson K 1998). Because GC B cells are in close proximity, higher-affinity cells might be able to pick up antigen from the surface of lower-affinity cells in direct cell-cell competition, prior to antigen internalization (Allen CD 2007b). Higher-affinity cells might be able to capture more antigens during rapid movement past the FDC processes. Furthermore, B cells may compete with antibody for access to antigens in IC on FDCs. This antibody initially will be natural antibody of low affinity. Later, this may be replaced by higher affinity antibody, raising the selection threshold. The higher affinity antibody may be either antibody arriving from outside the GC (Tew JG 1997), which is the hypothesis that has been tested in this thesis, or it may be antibody that is shed from centrocytes that have passed over and been selected on IC (Andersson K 1998). A further study recently showed that low affinity B cells in GC may have an intrinsically higher death rate (Anderson SM 2009), and so high affinity antibody in GC may act as a threshold and directly induce the relative low affinity B cells in the GC to undergo apoptosis.

To further prove the influence of different avidity IC for GC B cell selection, higher avidity IC was used. As expected, HiAv IgM^a-IC was apparent in GCs at day5 after immunization compared with LoMAv IC, confirming that avidity is a major factor for the antibody replacement in GCs. More interestingly, mice which received higher avidity IgM^a IC developed much smaller GC at d5 and d7, and fewer plasma cells in extrafollicular foci. It can be assumed, that the mutations introduced during the Ig hypermutation in centroblasts will lead to only limited variations in affinity. Raising the selection threshold too much in a short time will lead to negative selection of the vast majority of GC B cells. This may in the extreme case lead to complete abolition of the GC reaction, due to induction of apoptosis in most GC B cells. Mathematical modelling experiments, done in parallel to our experiments, lead to the same results (discussed later).

It has been noted previously that duration of the GC response and the availability of high affinity antibody or free antigen correlate. Bleeding of animals during an antibody response leads to a prolonged GC response (Tew JG 1980). According to the antibody feedback selection model this could be explained by the absence of antibody feedback in GCs leading to low selection pressure, resulting in a prolonged GC reaction. In a similar way, giving specific antibody during an immune response has been shown to dampen the immune response, which may be due to raising the GC selection threshold (Tew JG 1973). Therefore, antibody feedback may terminate the germinal centre reaction.

The response of anti-hapten in carrier-primed mice will be of low titre and affinity compared to a primary response, due to reduced thresholds of B cell activation during the recruitment of B cells into the responses, and free hapten in carrier-primed responses resulting in relaxed selection of centrocytes in GCs (Sze DM-Y 2002). This is the reason that soluble LoMAv antibody was detected in the GC after NP-CGG response in the

carrier-primed mice, not in the GC after the primary response to NP-CGG. As ELISA data has showed, LoMAv IC in the carrier priming will finally induce the same high affinity response as HiMAv IC and HiAV IC. So the carrier priming could expand the repertoire of B cells recruited, enabling them to respond faster upon encountering further variations of the pathogen, and the high finally antibodies in response will develop later. Conjugate vaccines are increasingly being used to raise immunity to bacterial capsule polysaccharide in infants, such as tetanus and diphtheria toxoids (Anderson 1983; Schneerson R 1983). The significant effects of carrier priming should be considered when designing immunization strategies.

3.3.5 Mathematical modelling of Ab production and GC reaction

The data described here reproduce predictions from *in silico* experiments of GC responses that were done in parallel studies. These experiments (M. Meyer-Hermann, A. Reynolds) were done by modelling 3D interactions and movements of GC B cells. Models were developed with or without a positive selection step where B cells have to compete with antibody in IC on FDCs. Furthermore this antibody was replaced over time with antibody of a similar affinity to GC output cells. Experiments were done where the affinity of the competing antibody in IC on FDCs at the beginning of the experiment were either low or high from the beginning (similar to our experiments described here).

The results from these *in silico* experiments showed that GC B cell affinity increased over time under all conditions. However, introducing a selection step that makes B cells compete with antibody on IC accelerated the appearance of positively selected high affinity mutant clones. The development of high affinity was further accelerated, if the affinity of antibody in IC varied over time and represented the affinity of clones leaving the GC after positive selection (A Reynolds unpublished). Varying the affinity of the

extraneous antibody present at the onset of the response had the effect that high affinity clones appeared earlier (around day 5 after the simulation), but this effect was lost during the next days (M Meyer-Hermann, unpublished) (Fig 3.22). These *in silico* studies were done to simulate what was done by us *in vivo*. High affinity antibody induces the earlier onset of high affinity GC output.

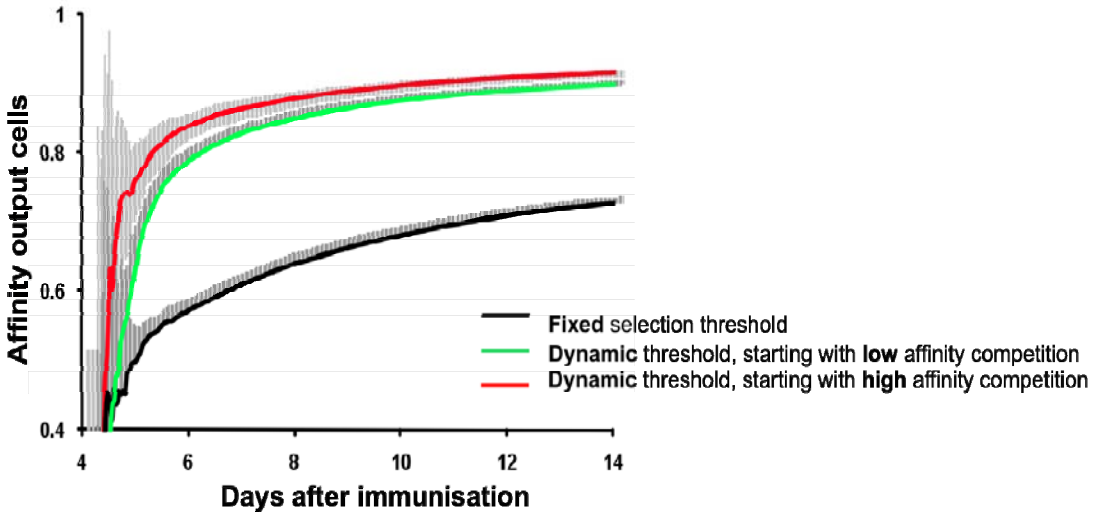

Figure 3.23: *In silico* simulation confirms that a dynamic increasing selection threshold is more efficient in producing high affinity antibodies
Independent *in silico* modelling study showing the effects of a dynamic selection threshold which is dependent on the affinity of antibody produced by plasma cells generated earlier in this GC.
- GCs with dynamic threshold produce high affinity output cells quicker and at higher affinity (compare coloured to black curve).
- High affinity antibody leads to higher affinity GC output, but this effect is strongest at the early stages of the response (compare red and green at day 5)

In silico experiments also modelled the size of GC reaction. In all simulations B cell number peaks at approximately 108 hours (about 4-5 days), and then reduces. The kinetics are quite similar to NP-CGG immunization of carrier-primed mice or primary TD antigens if immediate T cell help is available (Liu YJ 1991; Toellner KM 1996; Sze DM 2000). After low affinity antibody simulations, GCs remain until 360 hours. This response suddenly reduces and is truncated to 180 hours in the simulations containing

very high affinity antibody (Fig 3.23 A). The experiments have also shown that mutation rates are minimal when high affinity antibody is injected and that endogenous high affinity antibody does not develop significantly. The fraction of high affinity output cells and mutations per output cell is highest when an intermediated affinity antibody is injected. The highest rate of apoptosis per GC volume is predicted to occur when high affinity antibody is used for immunization (Fig 3.23 B) (Meyer-Hermann, unpublished). Finally Meyer-Hermman *et al* predicted that low affinity antibody would have minor effects on the kinetic of GC development, but very high affinity antibody injection would dramatically decrease the size of the GC reaction, and raise apoptosis levels. GC outputs would be very small, however, these outputs would be of higher affinity. This is exactly matched by what we found in our *in vivo* experiments. Smaller GCs and less fewer plasma cells in the red pulps were detected after injection with HiAv IgM-IC. In addition, the total titre of IgG was low, and the titre of high affinity IgG did not increase significantly, but the fraction of high affinity IgG was significantly higher (Fig3.14). The parallel mathematical modelling experiments further confirm that a dynamic selection threshold produces stringent selection for GC B cells, and induces more efficient affinity maturation early in the response.

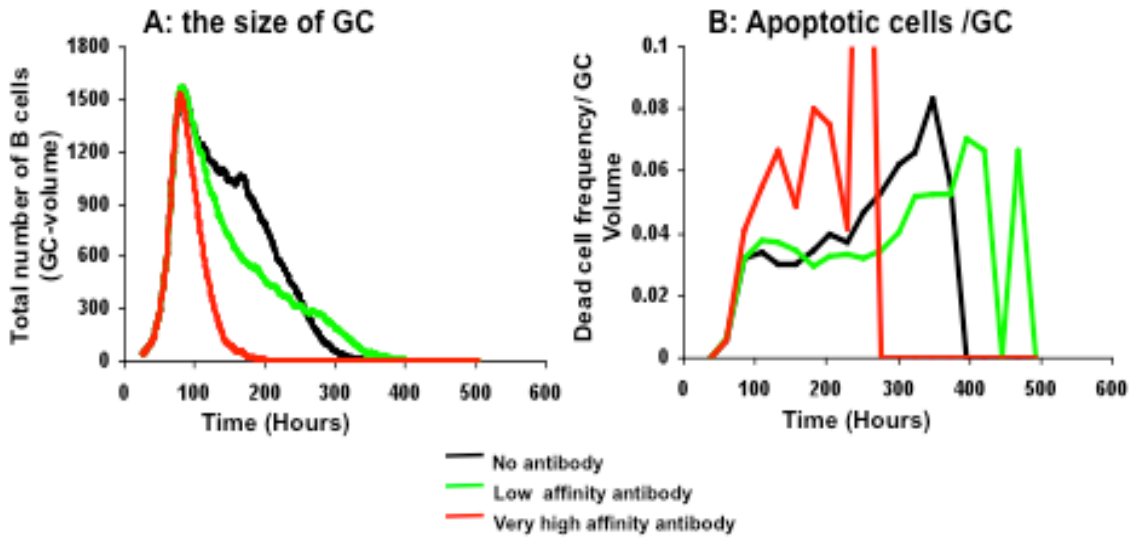

Figure 3.24: *In silico* simulation confirms that a dynamic increasing selection threshold is more efficient in producing high affinity antibodies
Germinal centers are not considerably affected by the injection of low to medium affinity antibody. However, injection of high affinity antibody stops the GC reaction by a strong induction of apoptosis. **A**: the kinetic of total number of B cells following the time, and (**B**) the kinetics of apoptotic cells at the same time point.

3.3.6. Effects of immune complex on the extrafollicular response

BCR affinity for antigen may control the B cell terminal differentiation pathways: activated B cells with low affinity BCR move preferentially into follicles to become GC B cells, whereas modest affinity clones become short-lived plasma cells (O'Connor BP 2006; Paus D 2006; Benson MJ 2007). The recent *in vivo* GC imaging reveals that GC seeding is an ongoing process and that only B cells with a competitive advantage in antigen binding can join a preexisting GC reaction (Schwickert TA 2007). A recent study showed that Fcα/μR, the Fc receptor for IgM on $B220^{+}$ spleenic cells, could mediate endocytosis of IgM-coated antigen by B cells (Shibuya A 2006). Therefore LoMAv IgM^{a}-IC may help increase the antigen-binding capacity of B cells, resulting in more activated B cells in response.

Some studies have provided evidence for affinity-independent cell entry into GCs. Where clonal competition occurs and higher affinity clones preferentially differentiate to plasma cells and memory B cells (Dal Porto JM 1998; Dal Porto JM 2002; Phan TG 2003; Paus D 2006).

Our experiments about the effects of antibody avidity on the extrafollicular response did not detect significant differences between groups after they received free NP-Ficoll or NP-Ficoll in the form of IgD/IgM-IC. This confirms that ICs mainly affect the GC response. Ehrenstein (1998) has compared the follicular response to protein antigen to the

extrafollicular response to TI-II antigen in mice that cannot make soluble antibody (sIgM). While the GC response is inhibited in the absence of sIgM, the extrafollicular response is increased when the epitopes are kept free (Ehrenstein MR 1998). Further the previous research reported that the administration of IgM complexed with T-independent antigens had no effect on antibody titre, duration or affinity maturation, despite increased antigen deposition in lymphoid follicles (Corley RB 2005). These results support the finding that antibody affinity in IC has a minimal effect for the extrafollicular response.

Haberman's review (2003) discussed the hypothesis that immune complex retention by FDCs is not required for germinal centre development. It is difficult to imagine how evolution of high affinity clones can happen without repeated contact with antigen. However, the role of antigen is not disputed in this review. Hannum (2000) shows that there is less antigen presented in GC, but there is also less antibody to cover epitopes, therefore the two effects may cancel out each other. Further, Germinal centres in mTg mice are much bigger than normal (Hannum LG 2000). Possibly there is less positive selection, and therefore bigger GC, as GC B cells are not leaving GCs, similar to that reported in $AID^{-/-}$ mice (Muramatsu M 2000).

3.3.7. Final outlook

Studies on hypermutation in germinal centres could be done to more directly show that the effects on antibody affinity observed are due to changes in GC selection during affinity maturation. EYFP expression in GC B cell of Cg1-Cre × ROSA eYFP mice can be used as a marker to easily FACS sort GC B cells. Germinal centres with less stringent B cell selection would be expected to show clonal relationships between individual GC B cells that produce genealogical trees that are less "bushy" than sequences GC B cells that have developed in under high stringency selection, while genealogical trees from GCs

with high selection pressure would have 'pruned' genealogical trees (Banerjee M 2002; Dunn-Walters DK 2002).

IgM^a-IC deposit on FDC may increase the GC B cell activation and affinity maturation by fixing complement and bridging BCR with complement receptor (CR1/CR2). Mice deficient in CR1/CR2 (Molina H 1996) can be used to study the role of different CRs compare to Fc-receptors for development of germinal centres. Specific complement receptors can be blocked with soluble CRs. We have recently received Ig-CR2 and will block C3 interaction to address this. Specific for antibodies to different variants of C3 and different CRs on FDC may be used to detect on FDC during different stages of the GC reaction, and the role for entry of immune complex into the spleen (van den Berg TK 1992). We can selectively inhibit immune complex trapping by FDC with monoclonal antibodies against C3. The role of Fcα/μR expressed on FDC may be tested by using $F(ab')_2$ fragment of different affinity during immunization.

An important aspect that has not been addressed is the role of Ig class switching for the regulation of the germinal centre reaction. Ig class switching produces a dramatic decrease in antibody avidity. At the same time IgG inhibits B cells activation binding inhibitory Fcγ receptors on B cells. The interaction between FDC, B cells, IgG and antigen are expected to be complex, but they may provide another mechanism to prolong the germinal centre reaction producing a lower selection threshold, once high affinity switched plasma cell clones have emerged from the germinal centre. Others have shown that pre-formed antigen-IgG1 complex can have effects on GC formation, clonotypic repertoire and stimulate somatic mutation in GC B cells (Nie X 1997; Song H 1998; Song H 1999). We have several NP-specific IgG hybridoma clones of different affinity and subclass (e.g. IgG3, IgG1) available to investigate their effect for B cell response.

Manipulating immune responses with affinity antibodies may be a useful way to accelerate affinity maturation in vaccine responses, either in situations, where a very fast onset of high affinity antibody production is desirable, or in aged individuals. Aged individuals have lower germinal center responses and problems with B cell selection in germinal centers, leading to reduced affinity maturation (Gibson KL 2009). The exact reasons are not clear, but if it is due to defects in production of antibody from germinal centre emigrant plasma cells, manipulation antibody responses in aged individuals with antibody competition would be a good way to increase GC selelction pressure and affinity maturation. Preliminary experiments on aged mice indicate that our immunization protocol may accelerate and improve affinity maturation in aged individuals. We will do further experiment to confirm this, and then plan to expand this to real pathogens. Targets would be vaccine antigens with little variation, where the production of such a complex and highly specific agent such as an antibody would make economical sense, and to provide fast acting vaccines for situations, where a very fast onset of affinity maturation is desired, as is the case after the onset of an epidemic.

Chapter 4 Plasmablast differentiation from Germinal Centres

4.1 Introduction

Antigen-activated B cells enter cell cycle in the T zone, and these B blasts subsequently differentiate into either plasmablasts that form extrafollicular foci at the junction between the red pulp and T zone, or GC founding cells in follicles (Liu YJ 1989; Jacob J 1991; Toellner KM 1996). A recent study from our group (Marshall, Zhang *et al* submitted for publication) analysed the gene expression associated with B blast differentiation by sorting single responding B cells on the basis of molecules expressed on the cell surface in QMxC57BL/6 mice after NP-Ficoll immunization at the different time points. The study showed that Bcl6 mRNA, but not protein, is expressed in naïve B cells. Bcl6 mRNA is repressed while B blasts move through the T zone 24-48h after immunization. At the same time *Aicda* mRNA is induced. This is lost 3 days after immunization when B blasts have moved into the red pulp, and express CD138. Transcription factors inducing plasma cell differentiation such as Blimp-1 and Xbp-1, and the mRNA for the downstream transcription factor Bob-1 are strongly induced. Finally Ig protein is produced in these $CD138^{+}$ plasmablasts. At the same time, GC founding cells appear in follicles, and re-express Bcl6 mRNA (and protein) and Bach2. Plasmablasts at this time completely lose expression of Bcl6, Bach2 and Pax5 mRNA (Fig 4.1).

IRF4 is a transcription factor that positively regulates the gene expression of *Aicda* and *Prdm1* (which encodes the Blimp-1), and also represses Bcl-6 to terminate the GC transcriptional programme (Sciammas R 2006; Teng Y 2007). It is not clear whether IRF4 acts as upstream of (Sciammas R 2006), in parallel to (Klein U 2006), or downstream of (Kallies A 2007a) Blimp1 in the generation of plasma cells. So far, all studies agree that IRF4 and Blimp-1 can increase XBP-1 transcription (Shaffer AL 2002; Klein U 2006), and so coordinate expression of Blimp1, IRF4, and XBP-1 regulates plasma cell

differentiation (Kallies A 2007b). Recent studies from our lab have shown that IRF4 is expressed at low levels in naïve B cells, and intermediately expressed in activated B cells when they enter the T zone after stimulation by NP-Ficoll in QMxC57BL/6 mouse. IRF-4 expression is lost when B blasts move into follicles and differentiate into proliferating centroblats. IRF4 can be found in a fraction of centrocytes which have been committed to differentiate into plasma cells, and is highly expressed in plasma cells (Falini B 2000; Marshall 2009) Thesis UoB). Low level expression of IRF4 may activate *Aicda* gene combining with Pax5 (Gonda H 2003), and Pax5 at this stage prevents Blimp-1 induction by promoting Bcl6 expression (Nera KP 2006). Increased IRF4 expression can inhibit Bcl-6 and induce Prdm1, and in turn, Prdm1 could feed back to enhance the IRF4 gene expression (Sciammas R 2006) (Fig 4.1). Theoretical studies suggest that low level of transcription factors like IRF4 could be critical for cells going through a phase of multilineage transcriptional priming (Laslo P 2006). IRF4 is required for the generation of plasma cells from both GC and memory cells (Klein U 2006; Jourdan M 2009). Even though it probably isn't critical for the generation and proliferation of memory B cells, IRF4 is expressed on memory cells (Cattoretti G 2006a), and may maintain memory cell pool (Klein U 2006).

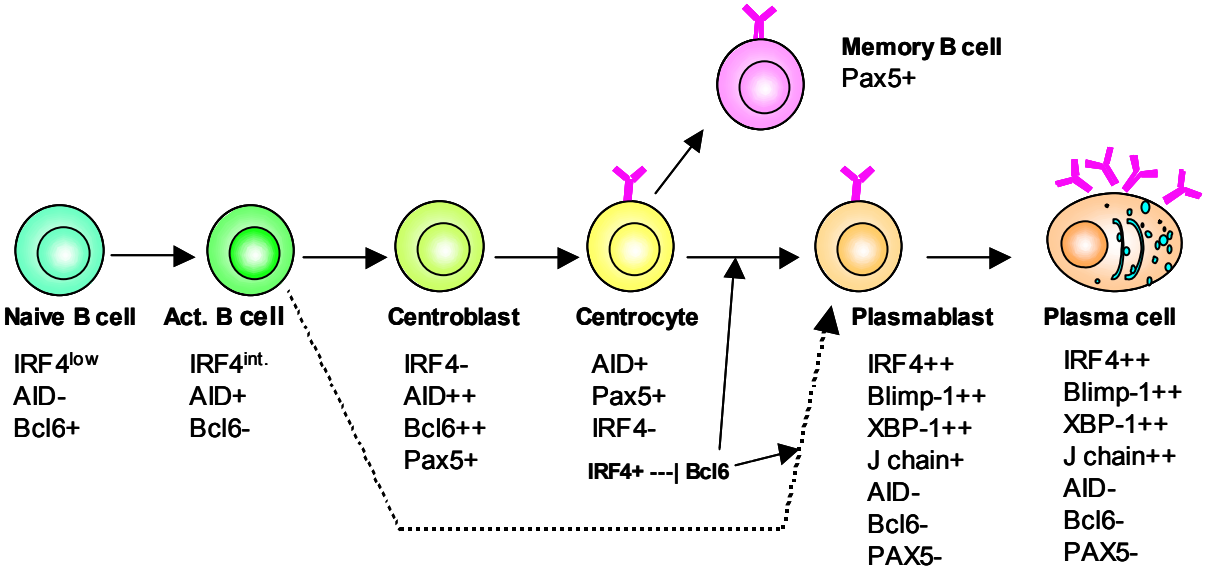

Figure 4.1: Plasma cell and memory B cell differentiation.
Changes in transcription programs that promote GC B cell differentiation towards plasma cell or memory B cell fate. Activated B cells move into the outer T zone, and then move back into follicles to form GCs, and then differentiate into plasmablasts. Alternatively they may directly differentiate into plasmablasts to form the early extrafollicular response. Int.: Intermediate. Act. B cell: Activated B cells.

In the classic model, activated B cells travel into follicles and form the GC. Germinal centre B cells will proliferate and undergo hypermutation in the dark zone, then move into the light zone, where they test the specificity of BCR by binding antigen in immune complex on FDC. This is followed by interaction with follicular T helper (T_{FH}) cells, which may lead to class switch recombination (Liu YJ 1996b) and to differentiation of high affinity antibody producing plasma cells or memory B cells (MacLennan 1994). It is not clear how successfully selected GC B cells move out from the GC: via the LZ, DZ, or from both (Fig 4.2).

In recent *in vivo* studies by multiphoton microscopy it was observed that cells in both light and dark zones have similar morphology and velocities, and transit occurs between dark and light zones in both directions in an apparently random way (Schwickert TA 2007; Allen CD 2007a; Hauser AE 2007a). These observations challenged the classical model in which GC B cells preferentially migrate from the dark zone to the light zone with recirculation back to the light zone, and have led to the proposal of alternative models of migration.

Studies on GCs from human tonsils had led to the conclusion that proliferation in GCs mainly occurs in the DZ. However, the recent work has shown that in murine GCs cells progress through the cell cycle in both DZ and LZ (Wang Y 2005; Allen CD 2007a; Hauser AE 2007a). Within both zones cells can be found going into S phase, with a higher frequency of cells in G2/M phase in the DZ (Allen CD 2007a; Hauser AE 2007a).

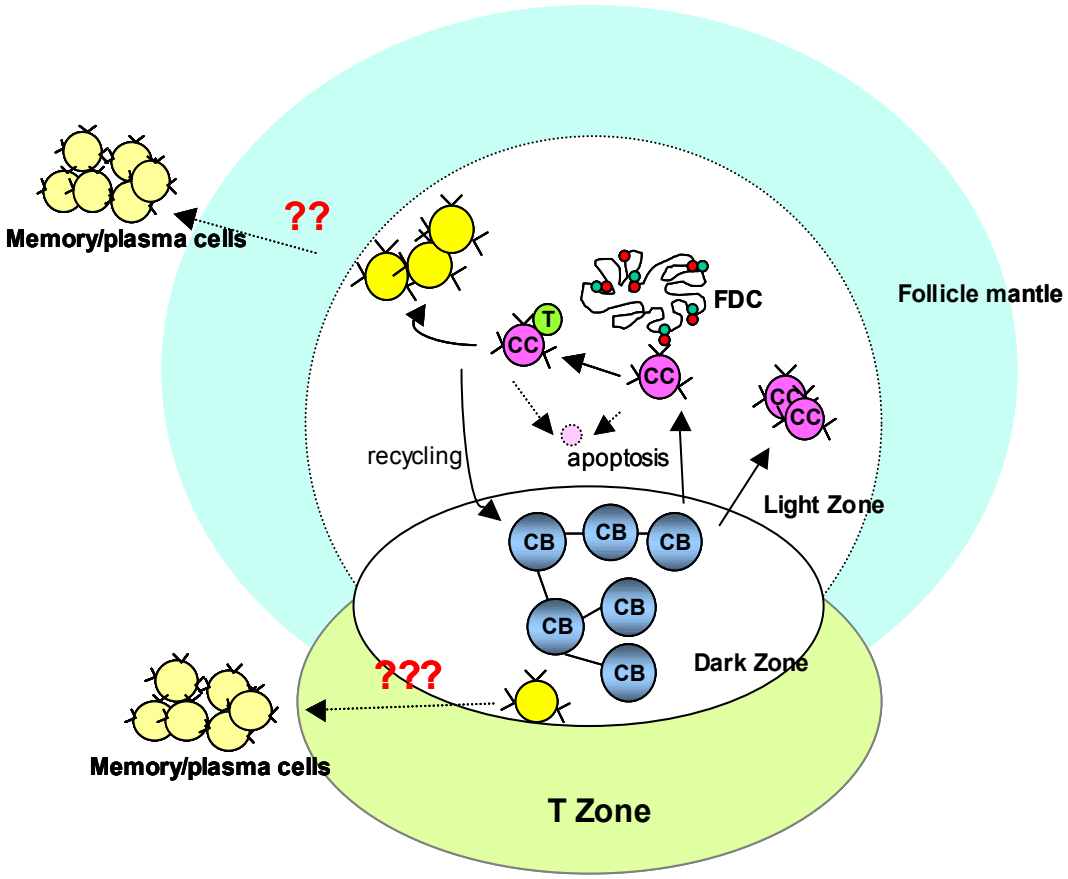

Figure 4.2: the B cell migration in Germinal Centre

In the classical GC model, centroblasts proliferate and undergo hypermutation in the DZ, and they then move into the LZ, differentiating into centrocytes. Centrocytes test their BCR by binging antigen on FDCs, followed by competing for signals from helper T cells. Cells will do apoptosis if they can't get signals from FDC or T help cells. Finally, successfully selected cells will move back into the DZ and do further recycling, or differentiate into plasma cells or long lived memory cells. The path, where selected cells take when leaving the GC, is not clear. LZ: Light zone, DZ: Dark zone. CB: Centroblast, CC: centrocytes, FDC: Follicular dendritic cell, T: follicle T helper cell.

Dependent on the time of immunization, proliferation and apoptosis can be seen in different zones of the GC. Early GCs can be seen as a focus of proliferating blasts inside follicles (MacLennan IC 1988). Separation into clear dark and light zone occurs later, with proliferating B cells in both (Fig. 4.3 A). Apoptotic cells and tingible-body macrophages are detected in dark and light zones (Fig 4.3B). Hauser et al (2007b) suggested that successive rounds of selection and mutation may occur within each zone, and also proposed the 'intra-zonal circulation model' where multiple rounds of selection and proliferation are required for the generation of high-affinity antibodies. Currently, it is not clear how these intravital studies should be interpreted and what the exact migratory processes in GCs are. A theoretical study reinterpreting intravital data showed that the observation periods of the intravital experiments are currently not long enough. So far the available intravital data does not exclude the traditional dark–light zone re-entry model (Meyer-Hermann ME 2006; Figge MT 2008).

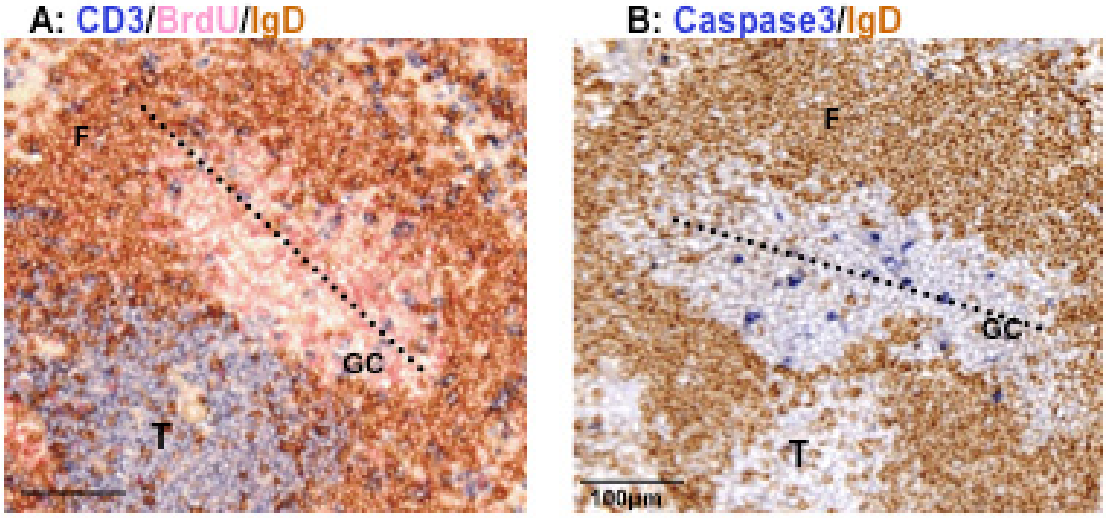

Figure 4.3: Proliferation and apoptosis happen in both light and dark zones
A: Spleen section from C57BL/6 mouse was stained with BrdU. The animal was immunized with SRBC, d5 it received BrdU 2hr before sacrifice. The light zone can be defined by the presence of T cells (Blue), and immune complex on FDC (light brown staining). Cells in both zones are undergoing proliferation ($BrdU^+$). **B**: spleen section, from d5 after NP-CGG response in the carrier-primed mouse, was stained for active Caspase3. Apoptotic cells appear in both zones. F: Follicle, T; T zone, GC: Germinal centre.

Follicle T helper cells and FDCs in the light zone may provide signals for cell division and selection. Further cells proposed to be important for selection are accessible for dark zone B cells either at the GC periphery or at the T-B zone interface (Lindhout E 1993; Fischer MB 1998; Haberman AM 2003). T-B zone interfaces are portions of the dark zone base in direct contact with T zone. The outer T zone contains activated T cells and DCs during the TD response (Grouard G 1996; Pape KA 2003; Okada T 2005). DCs in the T-B zone interface are theoretically capable of providing signals similar to those thought to be provided by FDCs, including presentation of retained, undegraded antigen (Wykes M 1998; Bergtold A 2005; Qi H 2006; Hauser AE 2007b). Low numbers of T cells can also be found in the dark zone (Arnold CN 2007; Haynes NM 2007). Studies in human tonsil found that T cells in the GC–T area interface express preformed CD40L similar to T_{FH} cells (Casamayor-Palleja M 1995). T cells in this area could play a role in GC B cell selection, providing that GC B cells would enter the T zone in this area. Real-time imaging observed the movement of GC B cells toward and away from the GC at the dark zone-T zone interface (Hauser AE 2007a).

The aim of this part of the project was to study the development and migration of plasmablasts leaving the germinal centre:

1) To determine the timing of IRF4 expression on B cells after stimulation
2) To identify the expression of IRF4 cells in different zones of the GC.
3) To confirm whether $IRF4^+$ cells on the GC-T zone interface are plasmablasts and whether they are derived from the adjacent GC.
4) To detect which costimulatory signals or cytokines may affect the generation of the early $IRF4^+$ plasmablasts on the GC-T zone interface by working on mice deficient costimulatory signals or cytokines.
5) To determine which signals are important for the production of the early plasmablasts

and locate sites of these signals on spleen section.

The main approach used was immunohistology to investigate the location and time of early IRF4$^+$ cells, and define the expression of these cells. Furthermore, mice deficient for cytokines and costimulatory molecules which may be involved in GC B cell selection were used to follow plasmablasts exiting from GCs. Histology, microdissection and RT-PCR were used in combination to define these signals and locate the site of their production.

4.2 Results

4.2.1 IRF4 is induced rapidly after B cell activation

To test the timing of IRF4 induction in B cells, QMxC57BL6 mice were immunized with NP-Ficoll. Surprisingly, IRF4 is induced rapidly after B cell activation. Using real time RT-PCR to detect IRF4 mRNA from whole frozen spleen sections shows that IRF4 mRNA levels peak within 1h after immunization and then quickly fall again, keeping at the intermediate level (Fig 4.4 A). FACS sorted B cells were analysed for their IRF4 mRNA expression. In naive B220$^+$ B cells low levels of IRF4 mRNA were found. IRF4 was strongly upregulated on B220$^+$NP$^+$FITC$^+$ (antigen binding) cells at 1h after immunization with NP-FITC-Ficoll (Fig 4.4B). Over the next hours mRNA expression levels fell again, and stabilized at intermediate expression levels at 4 hours. Finally, it was highly expressed when plasmablasts started to express CD138$^+$. The high expression level of IRF4 mRNA on activated B cells at 1h corresponds to the expression levels in plasmablasts.

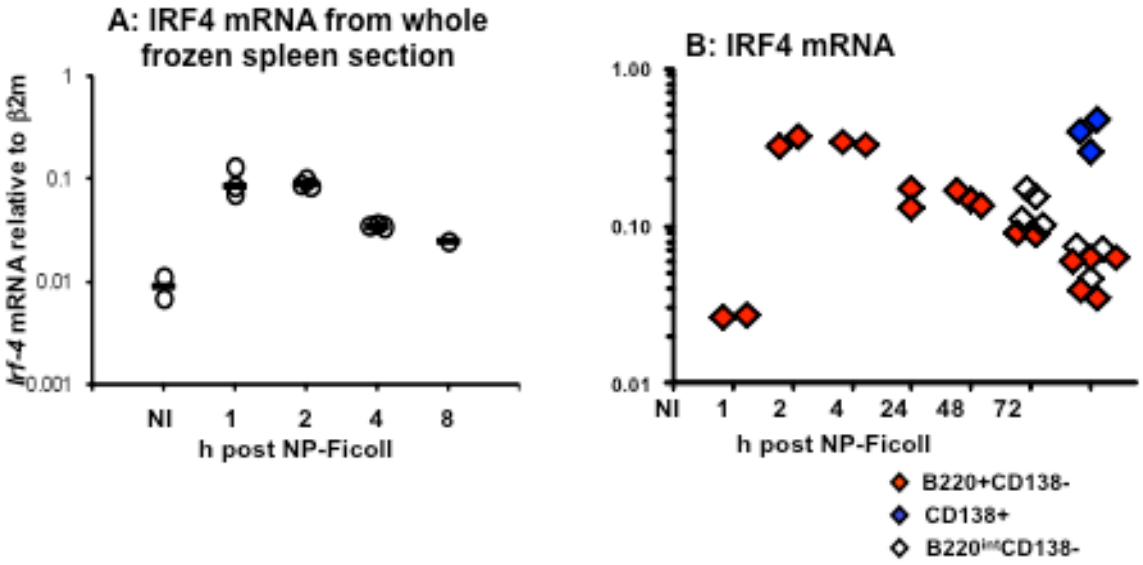

Figure 4.4: IRF4 mRNA expression after NP-Ficoll immunization
A: IRF4 mRNA level was measured in the whole frozen spleen section at the different time points after NP-Ficoll immunization in QMxC57BL/6 mice. **B:** Responding B cell populations from spleen sections were analysed for their expression of IRF4 mRNA at different time points after NP-Ficoll immunization. Each diamond represents one animal.

NP-Ficoll primarily activates MZ B cells and follicular cells via BCR, and upon activatin, B cells then move into T zone (Bajpai UD 2000; Vinuesa CG 2001). In non-immunized spleen sections, background NP specific plasma cells can be found in the red pulp. Naive NP binding B cells sit in the MZ and follicles (Fig 4.5 A). IRF4 staining shows that the same NP^+ plasma cells are IRF4 high, whilst MZ B cells are IRF4 low. Only a few individual $IRF4^+$ naïve cells travel in follicles. IRF4 protein is detectable in MZ B cells within 1h after NP-Ficoll injection (Fig 4.5 B). After 4h NP specific B blasts can be seen moving through the follicle, and some already appear in outer T zone, these B blasts express intermediate levels of IRF4 protein (Fig 4.5 C). 8h later, the outer T zone is filled with $IRF4^{int}$ B blasts, and these B cells express lower level of antigen binding (NP^+) than naïve MZ B cells, probably due to receptor blocking or internalization.

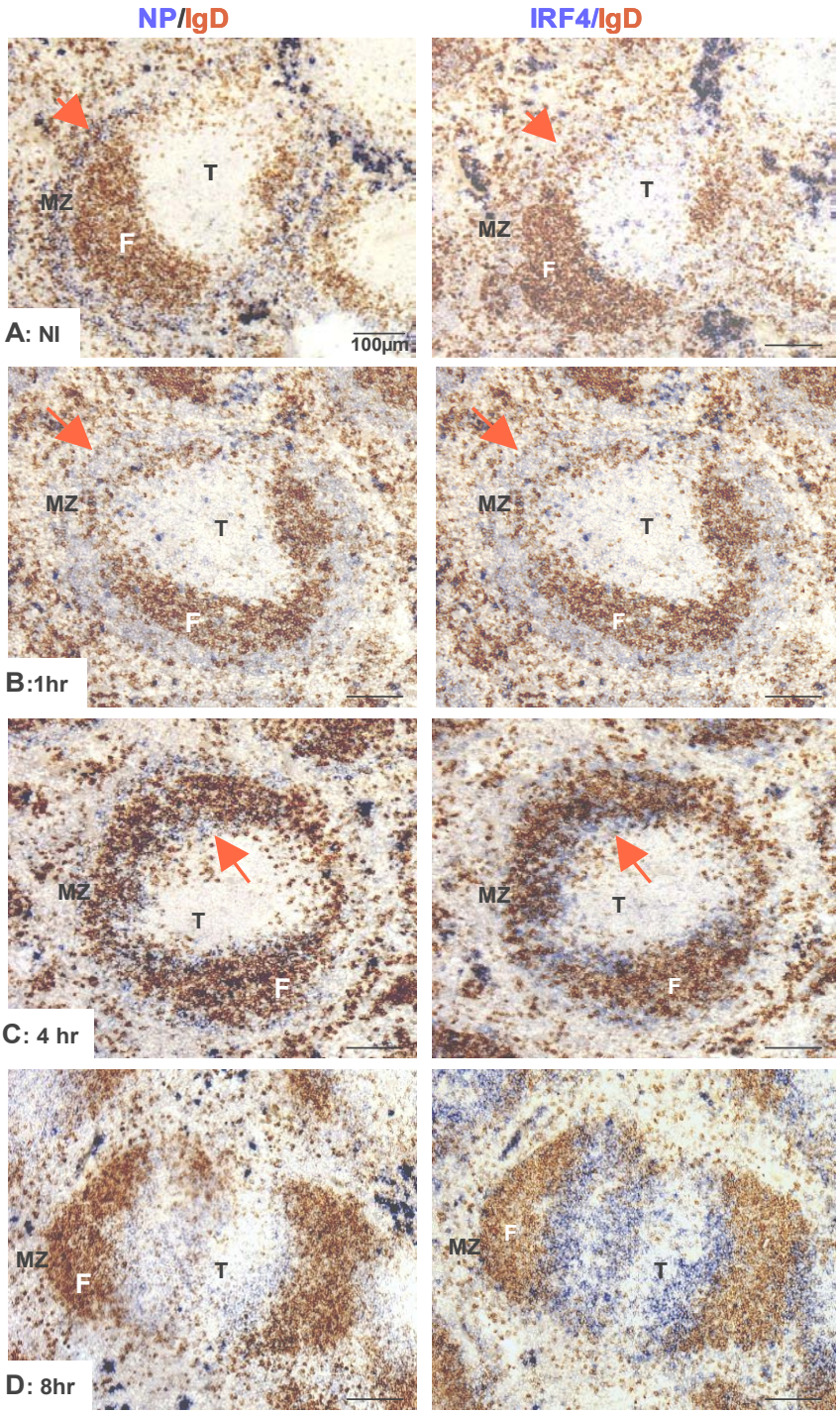

Figure 4.5: IRF4 protein induced shortly after B cell activation

Serial spleen sections from QMxC57BL/6 mice at the different time points after NP-Ficoll immunization were stained with NP/IgD (left) and IRF4/IgD (right). **A**: non-immunized section, **B**: 1hour, **C**: 4hour, and **D**: 8hour. F: Follicle. T: T zone, MZ: Marginal zone.

ImageJ software was used to quantify the IRF4 staining intensity on the tissue section at the different times after immunization (a detailed method is described in section 2.6.1). Usually more than 10 areas of the same size were chosen per individual tissue section. The mean IRF4 values (blue immunohistological staining) in different areas from one spleen section are shown in Fig 4.6 A (marginal zone), C (Follicle) and E (Outer of T zone) with the data from several mice in Fig B, D and F. IRF4 protein is detectable at very low levels in non-activated marginal and follicular B cells and at all times, expression levels were lower than in red pulp plasma cells. IRF4 protein increased above background levels within 1h after immunization in the areas, where NP-binding cells are found, particularly the marginal zones and follicles, and IRF4 expression reached peak levels in 2 hours, and then decreased (Fig 4.5 A B). Within the first 2 hours, the IRF4 expression level in the outer T zone was low, In the T zone IRF4 was seen from 4 hours after immunization, coincident with a reduction of the IRF4 level in marginal zones and follicles. This suggests that B blasts have arrived in the T zone. Double staining for NP-binding and IRF4 expression shows that all antigen-specific B blasts express intermediate levels of IRF4 protein (data not shown). Intermediate levels of IRF4 were maintained over the next 2 days on B blasts which reside in the T zone (data not shown). Three days after immunization, some B blasts differentiate into plasma cells expressing high level of IRF4, and some move into follicles to become GC founding cells that become IRF4 negative. Fig 4.7 shows a summary, comparing IRF4 expression in B cells of the MZ, T zone, red pulp and follicular location over the time course after NP-Ficoll activation.

These results strongly suggest that IRF4 can be used as a general marker for activated B cells. As IRF4 is involved in the regulation of AID expression, class switching and plasma cell differentiation (Klein U 2006; Sciammas R 2006), we chose IRF4 as a marker to investigate GC B cell activation and differentiation.

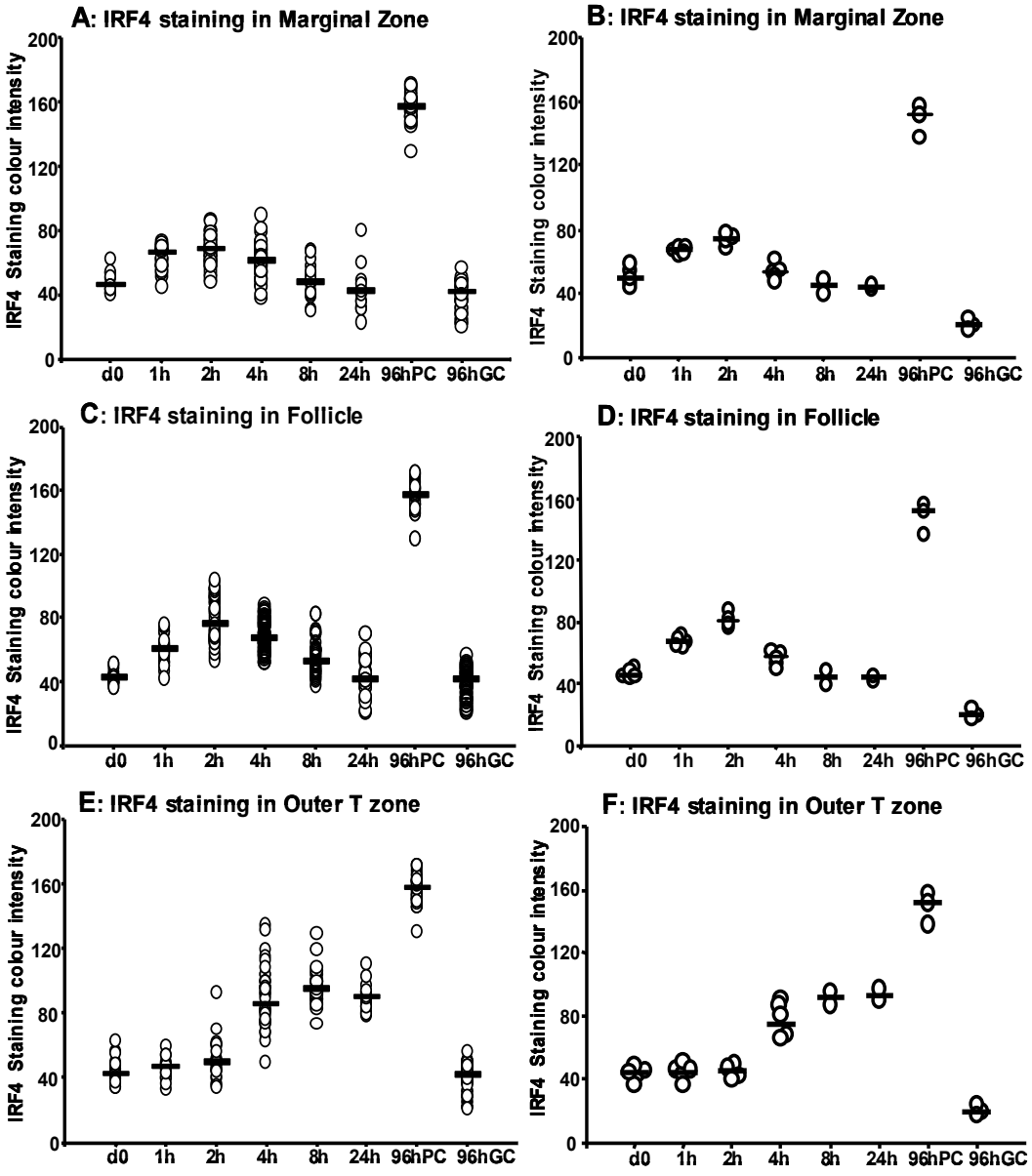

Figure 4.6: Quantification of IRF4 staining colour intensity

The amounts of IRF4 staining intensity in the marginal zone (**A, B**), follicle (**C,D**) and in the outer of T zone (**E,F**) at the different time point were shown. Each point in Fig A.C.E represents one interest region in the marginal zone, follicle, and outer T zone respectively. In Fig B. D. F. each point represents media data from one mouse. d0: non-immunization. 1h, 2h,4h, 8h, 24h, 96h: hours after NP-Ficoll immunization in QMxC57BL/6 mice.

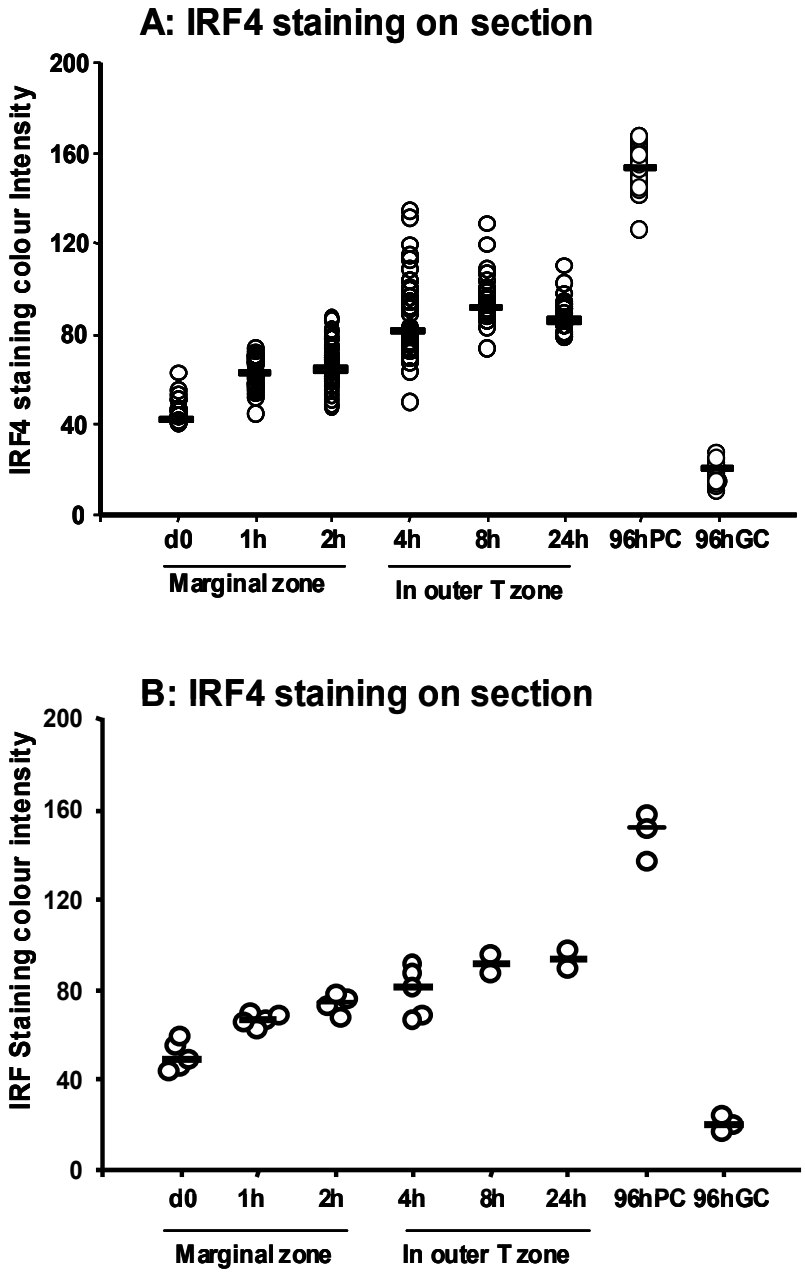

Figure 4.7: Summary of quantifying IRF4 staining colour intensity The amounts of IRF4 staining intensity in the marginal zone and in the outer T zone at the different time point were shown. Each point in Fig **A** represents one region in one spleen section. In Fig **B** each point represents media data from one mouse. d0: non-immunization. 1h, 2h,4h, 8h, 24h, 96h: hours after NP-Ficoll immunization in QMxC57BL/6 mice.

4.2.2 Appearance of IRF4$^+$ cells in the TD primary response

Firstly, to investigate the timing of IRF4 cells appearing in GCs in C57BL/6 mouse spleens, sheep red blood cell (SRBC) was selected as the antigen to induce GCs in primary antibody response. Injection SRBC in C57BL/6 mice induces a fast primary immune response in spleens. The kinetics are quite similar to the response in lymph nodes after footpad immunization with alum-precipitated NP-CGG (Toellner KM 1998). T cell priming starts around day 2, when IL-4 mRNA and IgG1 switch transcript above increase above background levels, this occurs slightly faster to what has been described for other TD responses (Luther SA 1997; Toellner KM 1998). IL-4 and IgG1 switch transcripts peak around d3 after immunization (Fig 4.8) before GCs have developed.

We used immunohistological staining to detect the timing of IRF4 expressing cells by studying the time course of the primary response. In non-immunized mice we found only very few cells expressing intermediate levels of IRF4 in follicles and T zones (Fig 4.9A). Apart from B blasts IRF4 is also known to be expressed in mature T cells, and it is upregulated upon CD3 stimulation (Matsuyama T 1995). IRF4 detected in the T zone of non-challenged spleens may be due to expression on occasionally activated T cells, similar to what is seen when naive lymph nodes are stained for BrdU (Luther SA 1997). Some background plasma cells expressing high level of IRF4 are seen in the red pulp.

At day3 after immunization, IRF4$^+$ cells were mainly clustered at the outer edge of the T zone (Fig 4.9 B). No GC or occasional small GCs are present in follicles. Cells in the T zone with IRF4 staining represent activated B cells and T cells. Immunofluoresence staining shows that IRF4$^+$ cells at the T zone-follicle interface represent both B cells and T cells (Fig 4.10B). Flow cytometry confirms that T cells upregulate IRF4 at day3 after immunization. This is reduced at later days (Fig 4.10A). This confirms that T blasts may interact with B blasts at this stage in the outer T zone, possibly via expression of CD40L

as proposed earlier (Casamayor-Palleja M 1995).

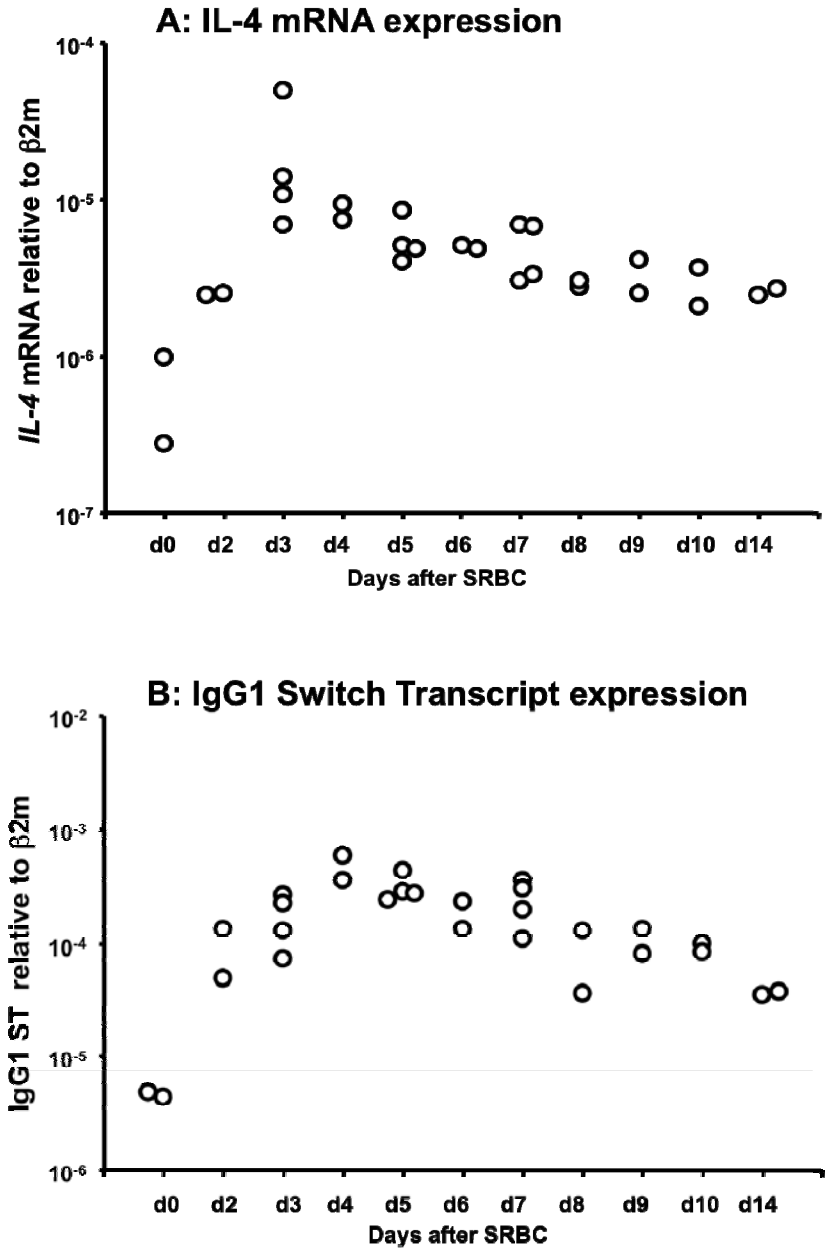

Figure 4.8: IL-4 mRNA and IgG1 switch transcript expression after SRBC immunization

(**A**) IL-4 mRNA and (**B**) IgG1 switch transcript expression level were measured in the whole frozen spleen section at the different time point after SRBC immunization in C57BL/6 mice. Each point represents one animal.

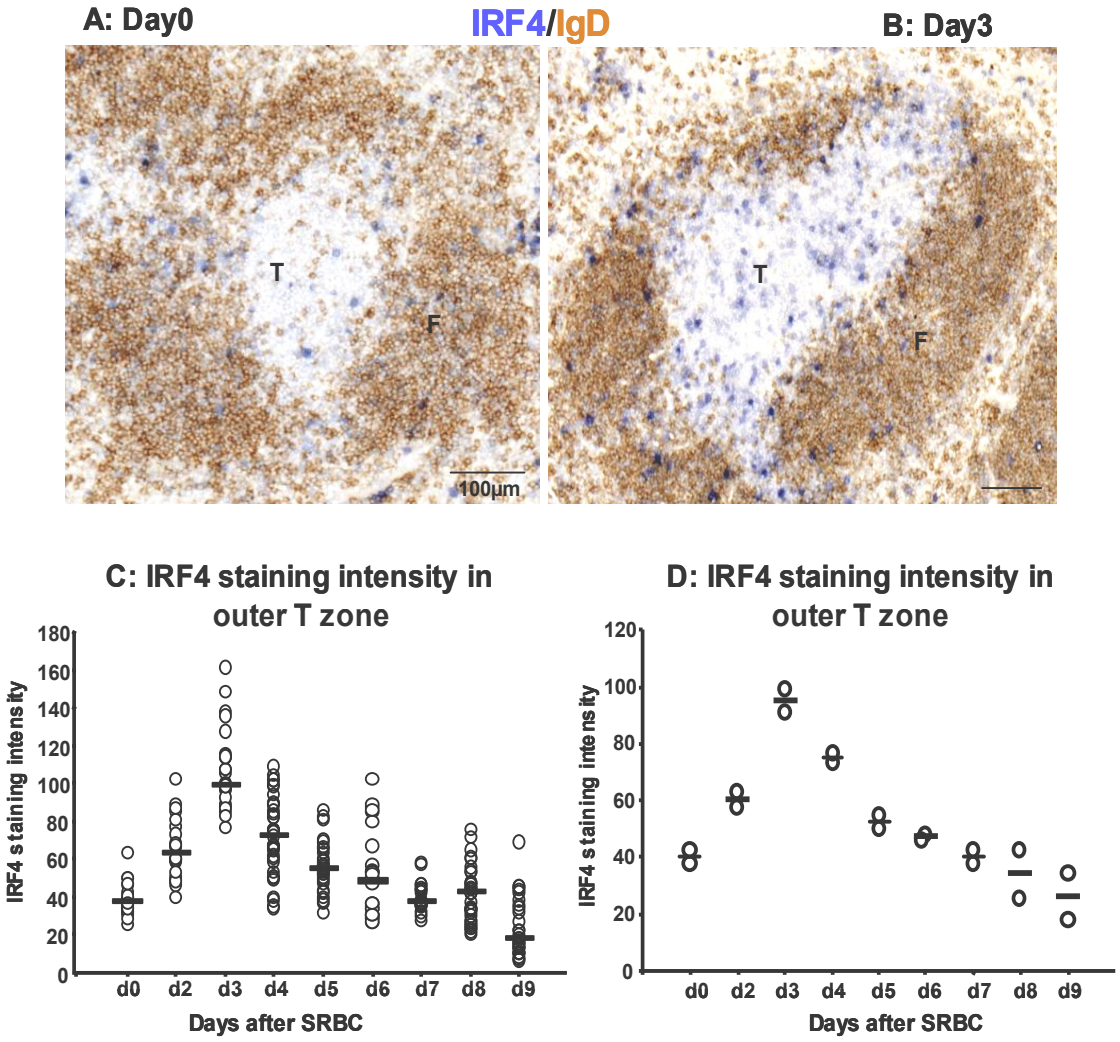

Figure 4.9: Timing of IRF4 cells in outer of T zone at the primary TD response
C57BL/6 mice were immunized with SRBC. **A**: d0 tissue section shows the IRF4 staining background. **B**: At day3, IRF4 expressing cells in the outer T zone. **C**: The quantification of IRF4 staining intensity shows that the maximum of IRF4 expressing cells accumulate at the outer of T zone at day3 after injection, and then reduce. Each point represents one area interest in one spleen section at each time point. **D**: a summary of IRF4 staining intensity in the outer of T zone, each point represents one animal. T: T zone, F: Follicle

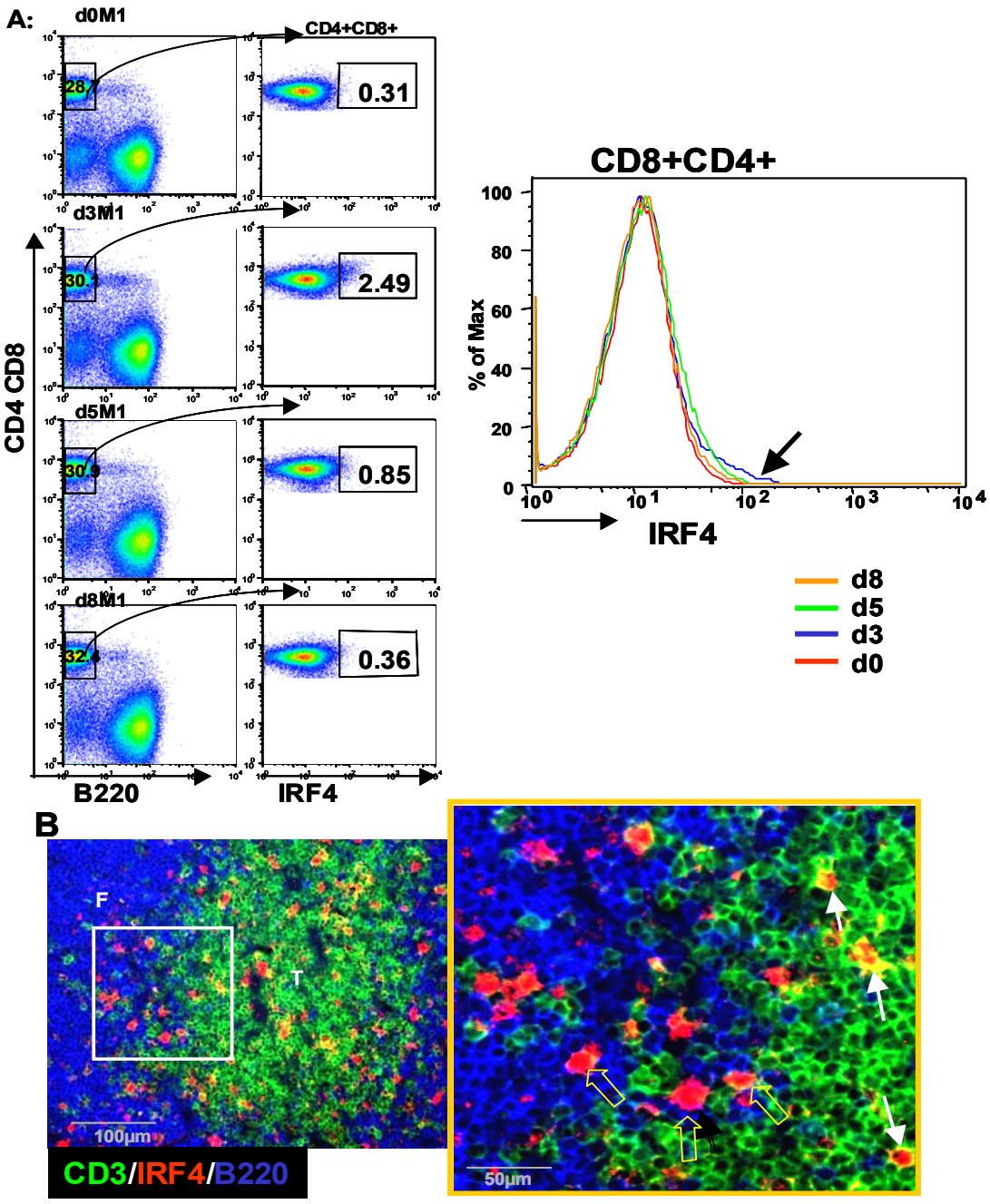

Figure 4.10: Activated T and B cells express intermediate level IRF4 at day3 after SRBC immunization

C57BL/6 mice were immunized with SRBC. **A**: At day3 FACS shows the upregulation of IRF4 on T cells, and downregulation at d5 and 8. **B**: Fluorescent staining further confirms that both activated T cells (small white arrow)and B cells (big yellow arrow) express IRF4 at the follicle- T zone interface. F: Follicle, T: T zone. This is representative of two individual experiment.

The quantification of staining intensity using ImageJ confirms the migration of small numbers of activated lymphocytes to the follicle-T zone interface at day2. By day3, IRF4 cells accumulated in this region, and then became fewer after day4 (Fig 4.9 C.D). This, together with data shown in Fig 4.10, confirms that, as shown in previous studies (Toellner KM 1998), the onset of T cell priming, and cognate interaction between activated B cells and helper T cells at this location occurs 3 days post-immunization.

Germinal centres were clearly seen at day 4 post-immunization. Surprisingly, $IRF4^+$ cells were first seen at the GC-T zone interface (Fig 4.11A). At day 5, maximal numbers of $IRF4^+$ cells were found in the GC–T zone interface (Fig 4.11B). These cells are usually located within the IgD^- areas that constitute the GC. The fluorescent staining on spleen section at day5 after NP-CGG immunization in the carrier-primed mice further shows that plenty of $IRF4^+$ cells at the GC-T zone interface already express CD138 and high levels of Ag-specific (NP+) Ig. These cells therefore appear to be plasmablasts (Fig 4.11C-E). At this stage IRF4 is mainly expressed in B blasts and plasmablasts, not in T cells (Fig 4.11C). This is confirmed by flow cytometry staining (Fig 4.10A).

Around 6 to 7 days after immunization, germinal centre has reached maximal size and the numbers of $IRF4^+$ cells seen at the GC-T zone interface declined (Fig 4.11E.F). At day9, the numbers of $IRF4^+$ cells at the GC-T zone interface appeared to decline further (Fig 4.11F and H). By day 14, GC size became smaller, and just few $IRF4^+$ cells presented at the GC-T zone interface. It seems likely that these $IRF4^+$ cells are early plasmablasts leaving the GC at an early stage via the dark zone–T zone interface, and then migrating through the outer T zone and develop into plasma cells in the red pulp (Fig. 4.11C-E).

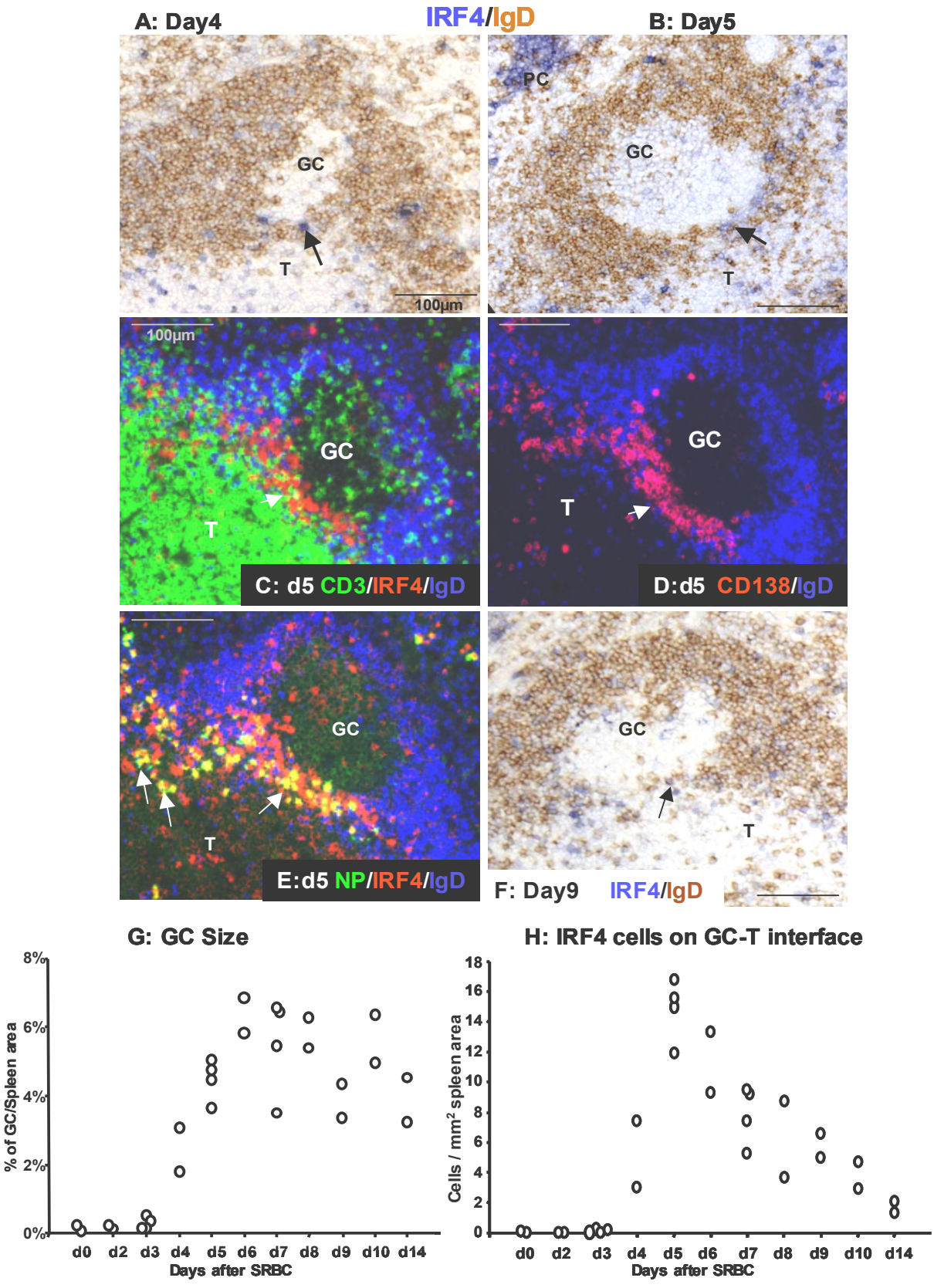
A: Day4
IRF4/IgD
B: Day5
GC
T
100μm
PC
C: d5 CD3/IRF4/IgD
D:d5 CD138/IgD
E:d5 NP/IRF4/IgD
F: Day9 IRF4/IgD
G: GC Size
% of GC/Spleen area
8%
6%
4%
2%
0%
d0 d2 d3 d4 d5 d6 d7 d8 d9 d10 d14
Days after SRBC
H: IRF4 cells on GC-T interface
Cells / mm² spleen area
18
16
14
12
10
8
6
4
2
0

Figure 4.11: Timing of appearance of IRF4$^+$ cells at the GC-T zone interface at the TD primary response

C57BL/6 mice were immunized with SRBC and then the GC size and IRF4$^+$ cells at the GC-T zone interface were measured by staining IRF4 (blue) and IgD (brown). **A**: IRF4$^+$ cells appear at the GC-T zone interface at d4 just after GCs form. **B**: Plenty of IRF4$^+$ cells present at the GC-T zone interface at d5. Fluorescent staining on the spleen section from d5 after NP-CGG response in carrier-primed mice shows that these IRF4$^+$ cells are not T cells (**C**), upregulate CD138 (**D**) and NP-specific antibody, related to the adjacent NP$^+$ GC (**E**). **F**: day9 the number of IRF4 cells at the GC-T zone interface reduced. **G**: the GC development during the day time course after immunization, **H**: the timing of IRF4$^+$ cells appeared on the GC-T zone interface after immunization. T: T zone, F: Follicle, GC: Germinal centre, PC: Plasma cell. NP-CGG immuzation can help detect antigen-specific plasamblasts.

While at day10 the numbers of IRF4$^+$ cells seen at the GC-T zone interface reduced further, considerable numbers of IRF4$^+$ cells were found in the LZ (Fig 4.12A). Immune complexes on FDC easily produce non-specific staining. Counterstaining with IgM confirmed that the IRF4 staining seen in the LZ was specific staining (Fig 4.12B). The quantification for the appearance of IRF4 expressing cells in LZ showed that only a few IRF4 cells were seen at day5, and more cells appeared at the later response. The number of IRF4 cells in LZ reached the peak at day7, and remained until day10 (Fig 4.12C). Four colour fluorescent staining of day8 samples showed that while some IRF4$^+$ cells were still located on the GC-T zone interface (Fig 4.14 A D: small white arrow), many IRF4$^+$ cells now presented in the LZ, particularly in the area adjacent to the follicular mantle where the majority of T_{FH} cells localize (Fig 4.13A B C white line). High power magnification showed that most IRF4$^+$ B cells were often associated with T cells, possible interacting and receiving signals from T cells (Fig 4.13 B C: big white arrows). Additionally, some T cells express IRF4 in both LZ and GC-T zone interface. These possibly represent antigen-specific activated T cells. IRF4 has been reported to have a function in T helper cell differentiation promoting expression of IL-4 and IL-10 (Rengarajan J 2002; Ahyi AN

2009), which stimulate B cell differentiation and antibody production (Briere F 1994; Toellner KM 1995).

IRF4 expression associated with centrocyte activation in the LZ may be a different development phase of B cell differentiation in the GC. It may be associated with memory B cell activation or plasma cell differentiation, as some $IRF4^+$ cells in the LZ upregulate CD138 (Fig 4.13C and Fig 4.14 D, yellow arrow). Taken together, IRF4 expression cells seen in cells in the LZ and GC-T zone interface might indicate that there are two pathways of GC B cell leaving GC from either LZ or DZ.

In follicles, two waves of $IRF4^+$ cells were observed at different times after immunization (Fig 4.11, Fig. 4.14). Quantification shows that, as mentioned above, naive follicles contain considerable numbers of IRF4 expressing cells. IRF4 expressing cells became fewer at day2, when activated blasts were seen in the T zone. At day 3, more IRF4 expressing cells were seen again in follicles. This stage probably corresponds to B blasts undergoing rapid clonal expansion at the perimeter of follicles, where they become GC feeding cells (Coffey F 2009). At this stage, EBI2 (Epstein-Barr virus–induced gene2) expression promotes B cells to move to the outer follicle and inter-follicular regions (Gatto D 2009; Pereira JP 2009). By day5, when GCs have formed, IRF4 expressing cells reduced in the follicles, but became more abundant after selection of centrocytes in the GC light zones had started, around day 8 (Fig. 4.14). This could correspond to the exit of the high affinity plasma cells and/or early GC-derived memory B cells, as described. Anderson *et al* showed that memory B cells localize in the outer follicle (Anderson SM 2007), and IRF4 is known to be expressed on memory B cells (Klein U 2006). The results of the timing of IRF4 appearance in the LZ indicate that stable and long-term GC output cells may exit from the LZ, though follicles. Also these observations highlight that the peripheral GC border may be a substantial component of the migratory route.

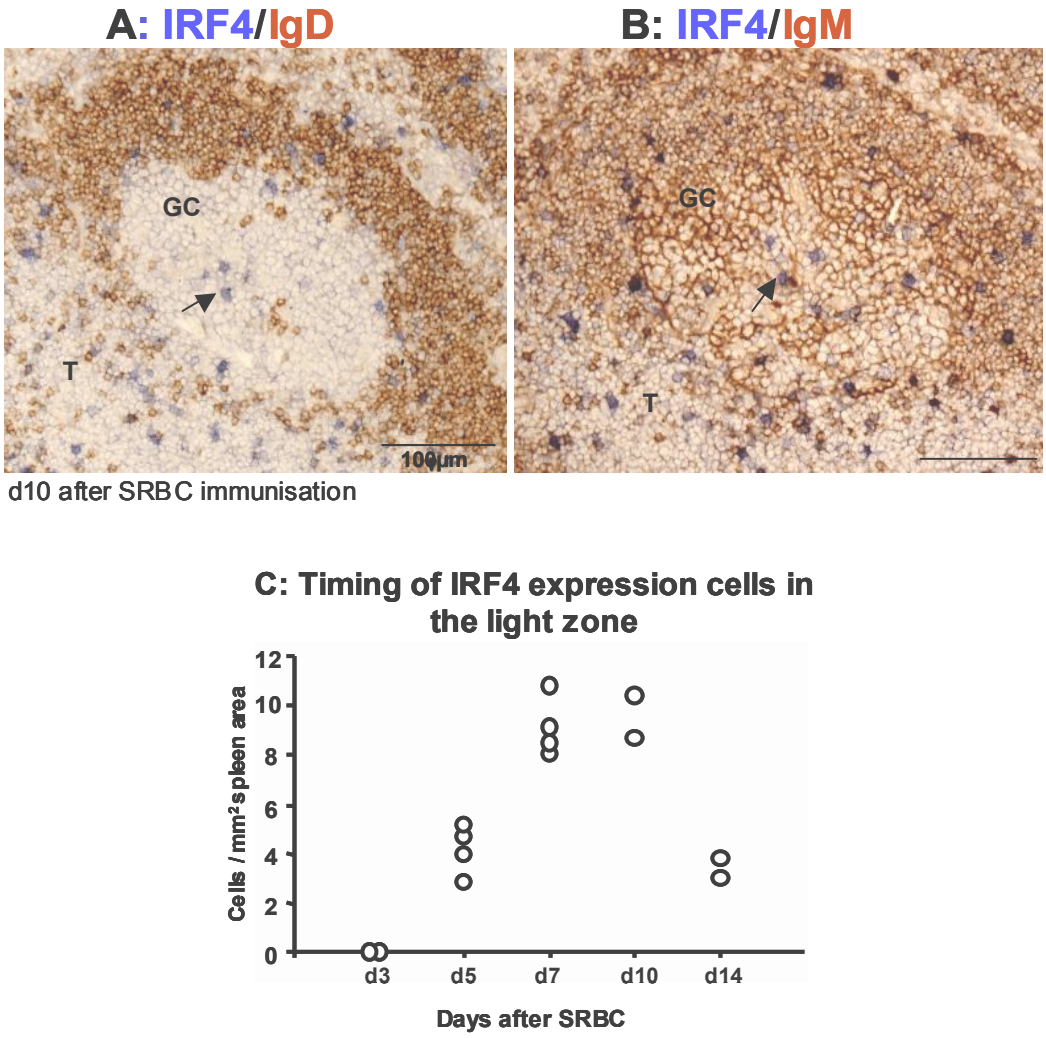

Figure 4.12: Timing of IRF4$^+$ cells in light zone at the primary TD response
C57BL/6 mice were immunized with SRBC. **A**: At day 10, IRF4+ cells clearly presented in the light zone. **B**: IgM as counterstaining further confirmed that these cells in the LZ are IRF4+ not non-specific staining on immune complex. **C**: the quantification data shows the timing of IRF4+ cells in the LZ after immunization. Each point represents one mouse. GC: Germinal centre, T: T zone.

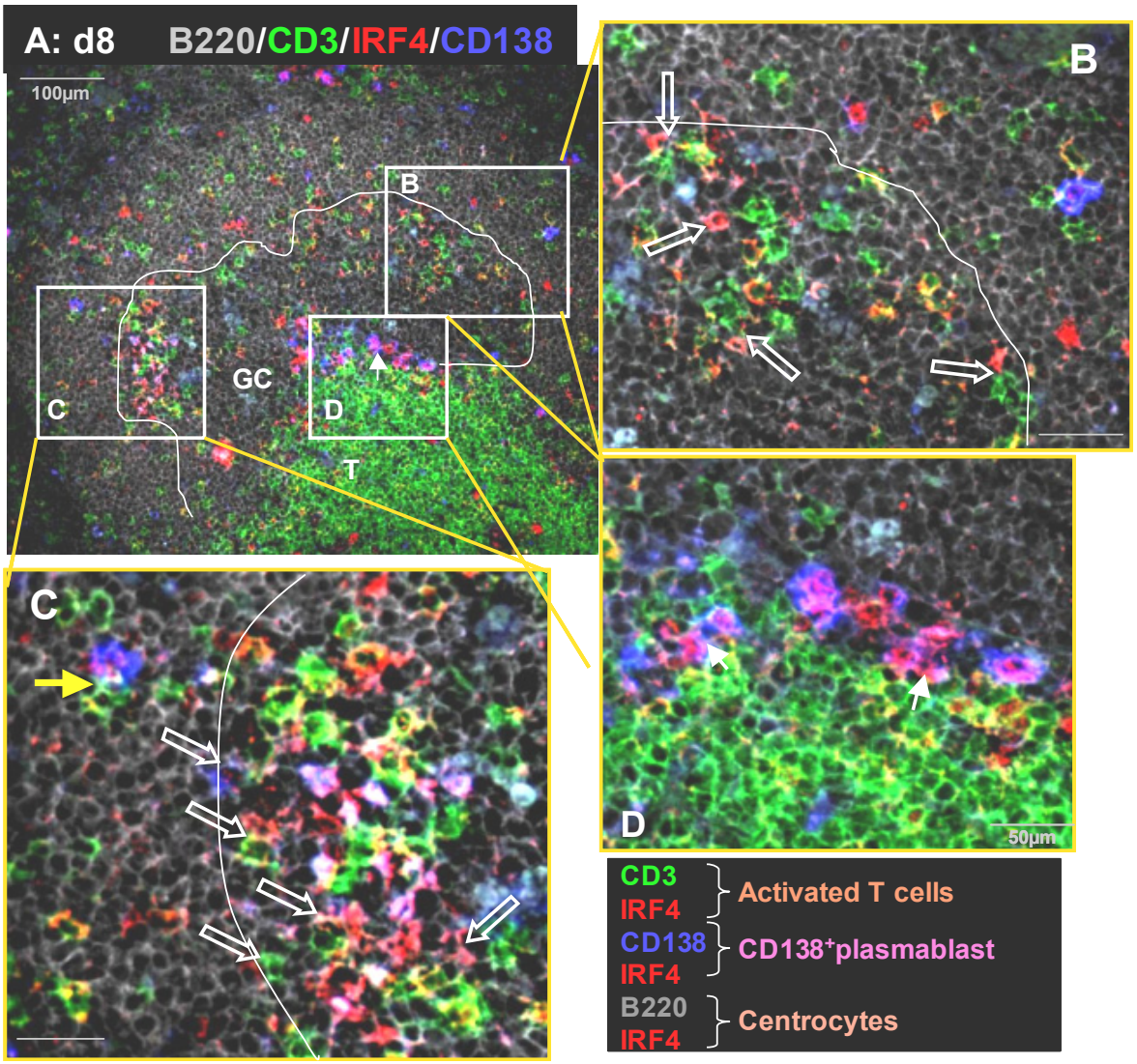

Figure 4.13: IRF4$^+$ B cells in light zone at day 8 after the primary TD response
Fluorescent staining on the spleen section from C57BL/6 mice d8 after immunization with SRBC. **A**: follicle containing GC and T zone. IRF4+ cells at the GC-T zone interface (small white arrow), and in the LZ close to the follicle mantle (white line). **B** and **C** with high power magnificent show some IRF4+ cells closely localised with follicle T help cells in the LZ (big white arrows). **D**: IRF4+ B cells at the GC-T zone interface. GC: Germinal centre, T: T zone

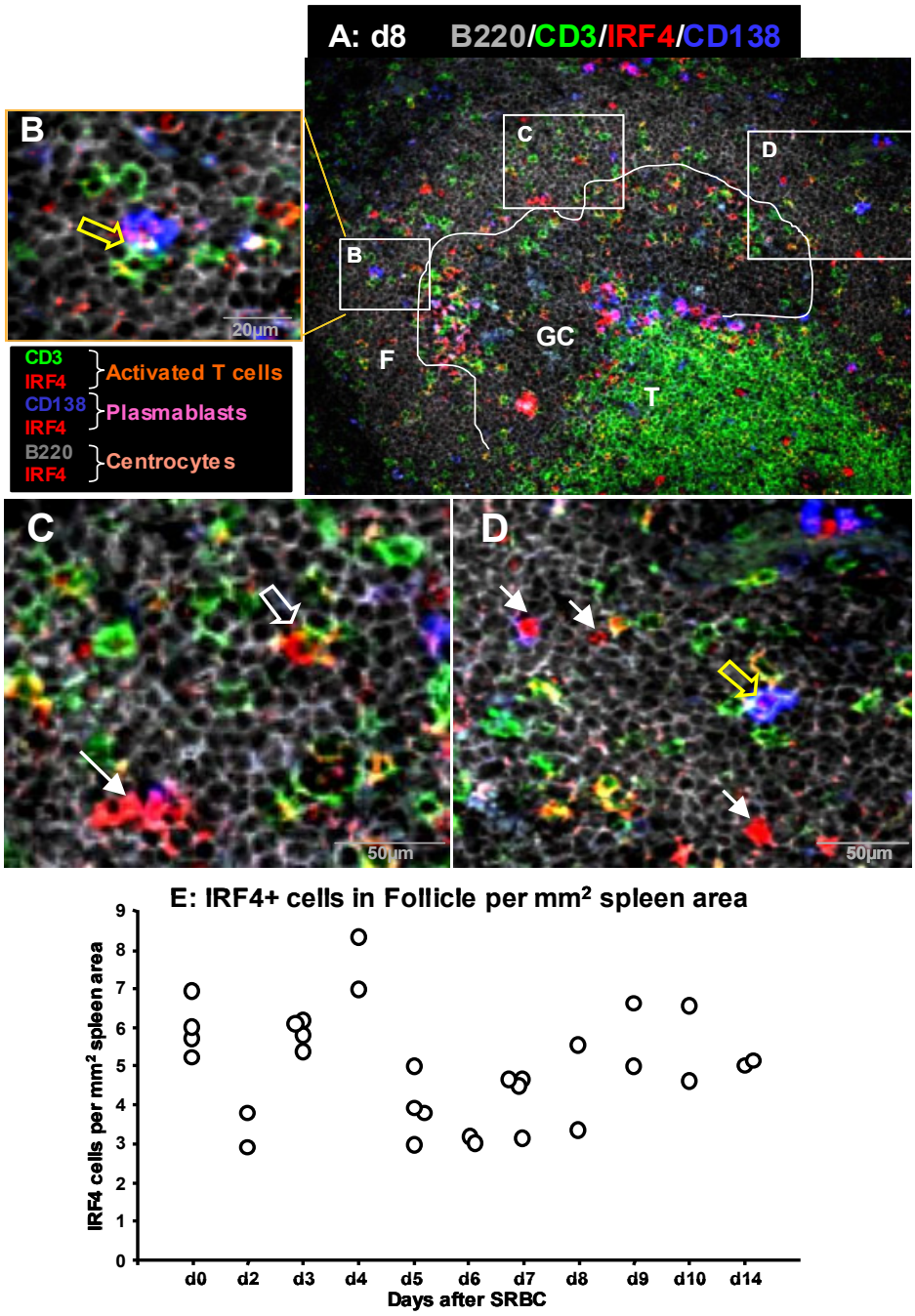

Figure 4.14: Timing of IRF4 expression cells in follicle at the primary TD response

IRF4 expression cells present in follicles before and after immunization. **A**: Fluorescent staining shows IRF4+ cells in the follicle from d8 after immunization with SRBC in C57BL/6. **B**, **C**, **D**: high power magnificent show IRF4+ cells in the follicle, some are CD138+ (big yellow arrow), some are not (white arrow). **E**: The quantification data shows the timing of IRF4 expression cells in follicles. Each point represents one animal. GC: Germinal centre, T: T zone, F: Follicle

The data of the appearance of $IRF4^+$ cells in different zones and the possible migration routes are summarized in figure 4.15. In summary, naïve B cells expressing intermediate level of IRF4 recirculate through follicles. After stimulation, B blasts with intermediate levels of IRF4 expression start to move to the B-T boundary, where they divide and meet antigen-specific T cells. After accumulation in this region, B blasts migrate back to the outer follicles, where they may undergo further clonal expansion, and become GC founding B cells. By day5 most B cells have joined the GC reaction. Just after GCs have formed, early GC-derived plasmablasts with higher levels of IRF4 expression appear at the GC-T zone interface. These cells travel through the outer of T zone into the red pulp, and differentiate into early GC derived plasma cells. From day6, IRF4 expression is detected on the light zone B cells. This may be a different developmental phase, involving prolonged hypermutation, and may require a different selection processes on FDC and T_{FH} cells. This may lead to the production of long-lived GC-derived high affinity plasma cells and memory cells for a long-term humoral response. This should be further investigated, for example, by the use of different markers such as CD138 for plasma cells, or CD38, CD80 and Pax5 for memory cells. In addition, chemokine receptors such as CXCR4 and CXCR5 could be utilise to further identify differentiation and migration of $IRF4^+$ cells in the LZ and the follicle mantal at the later stages of the response. In this thesis I mainly focus on the characterization of IRF4 expressing cells leaving the early GC from the GC-T zone interface.

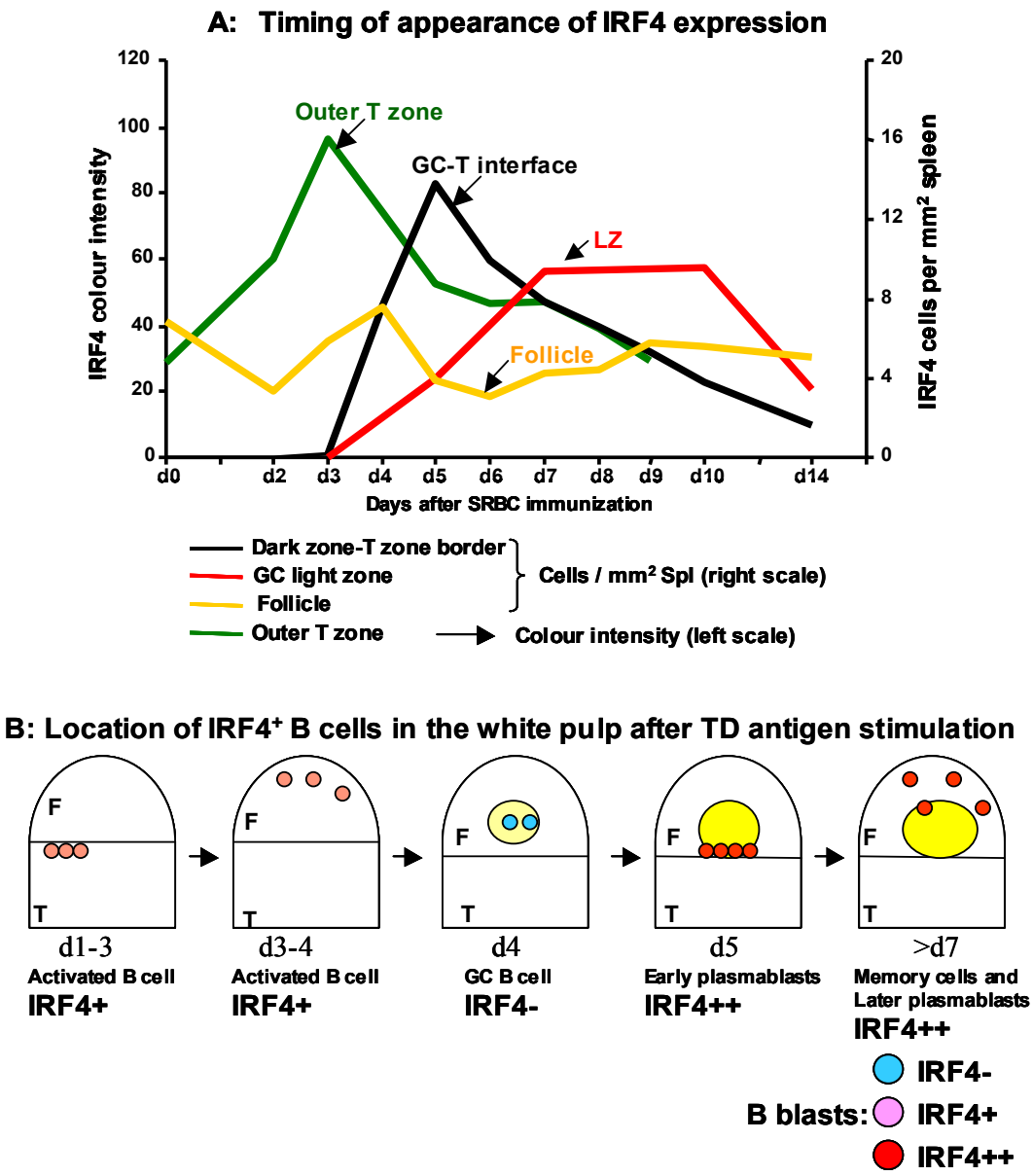

Figure 4.15: Timing of IRF4 expression in different splenic microenvironment

A: The summary of the timing of IRF4 expression cells in different areas of the white pulp after SRBC immunization in C57BL/6 mice. IRF4 expression cells in follicles, the LZ and the GC-T zone interface are presented per mm2 spleen area (right scale), IRF4 expression cells in the outer T zone is assessed by quantifying staining colour intensity (left scale). **B:** After SRBC injection naïve B cells get activation and differentiation into B blats expressing intermediate level IRF4 (IRF4+), move into the outer T zone. After contract with T cells, they migrate back into the follicle to expand and become GC founding cells and lose IRF4 (IRF4-). Just after GC forms, early plasmablasts expressing high levels of IRF4 (IRF4++) appear at the GC-T zone interface, whilst in mature GCs, B cells may get preferentially selected in the LZ and leave through follicles.

4.2.3 Characterization of IRF4 expressing cells on GC-T zone interface

To identify the differentiation stage of $IRF4^+$ cells at the GC–T zone interface we used a selection of markers typical for plasma cell and GC differentiation. All studies were performed in early stage GCs, 5-6 days after immunization with SRBC. .

Cg1-Cre xROSA-eYFP mice were used to identify the presence of cells that have undergone through induction of IgG1 switching, as B cells which have undergone this process, become eYFP positive (Fig. 4.16). Typically, most GC B cells appear yellow, and eYFP expression shows that a B cell is likely to be derived from GCs (Casola S 2006). Three days after SRBC immunization individual $eYFP^+$ cells appear in the outer T zone and the inner follicle (Fig 4.16A). This represents extrafollicular T–B cell interaction (as discussed above). By day4, activated B cells have already differentiated into extrafollicular plasmablasts in the junction between T zone and the red pulp, and into GC B cells in follicles (Fig 4.16 B). Around 40% of these plasmablasts, which have developed outside follicles, are eYFP positive (data not shown). At day6, large GCs (and larger plasma cell foci) have developed. GC B cells express lower levels of eYFP (Fig 4.18C). Very obviously, cells with high level of eYFP expression have appeared at the GC-T zone interface. These cells are $CD138^+$ and IgG1 switched (Fig 4.17 A B). High frequency of eYFP expression in extrafollicular foci (data not shown), similar to that seen in adjacent GCs, shows that these cells are GC derived.

Blimp-1 acts as a master regulator to drive terminal differentiation of mature B cells into plasma cells (Turner CA Jr 1994; Shaffer AL 2002). A Blimp-1EGFP reporter mouse (Ohinata 2005) was used to identify Blimp-1 expression after immunisation with SRBC. (mouse spleen tissue was kindly donated by Dr. M. Turner Cambridge UK). Interestingly, cells expressing Blimp-1 (green) were seen at the GC-T zone interface and in the red pulp 6 day after immunization, but not in GCs (Fig 4.17C D). At the same time, these

Blimp-1EGFP expressing cells also upregulated CD138 and IRF4. Similar results showing Blimp-1 expressing cells at the same location have been presented (Angelin-Duclos C 2000). Immunostaining showed that most of the IRF4 cells on the GC-T interface expressed switched IgG1 and IgG2a, with very few cells still IgM positive (Fig 4.17E F), and most cells switched to IgG1. These shows that $IRF4^+$ cells on the GC-T zone interface have irreversibly started terminal differentiation to the plasma cell phenotype.

Next, we tested markers expressed by GC B cells to study whether $IRF4^+$ cells on the GC-T zone interface have lost GC identity. GL7, originally identified as late activation antigen on B and T lymphocytes (Laszlo G 1993; Luzina IG 2001), is commonly used as a GC B cell marker (Han S 1996). IRF4 expressing cells on the GC-T zone interface are completely GL-7 negative, while few $IRF4^+$ cells in the LZ are still $GL\text{-}7^+$ (yellow arrow)(Fig 4.18A). Intermediate levels of Fas are expressed on GC B cells (Liu YJ 1995; Martinez-Valdez H 1996), while many plasma cells express high levels. $IRF4^+$ cells on the interface co-express high levels of Fas at a similar frequency to plasma cells in the red pulp (Fig 4.18 B). The expression of Bcl6 protein regulates GC development and function (Cattoretti G 1995; Allman D 1996). Post-GC B cell development towards plasma cells requires Bcl-6 downregulation (Basso K 2004). IRF4 cells on the GC-T zone interface are completely Bcl6 negative (Fig 4.18C). PNA binds strongly to B cells in GCs (Jacob J 1991). Histological staining shows that PNA is strongly expressed on GC B cells, and it is absent on $IRF4^+$ cells localized at the G-T zone interface, while $IRF4^+$ centrocytes do express PNA (yellow arrow) (Fig 4.18 D). Complement receptors CD21/CD35 are expressed primarily on B cells and FDCs (Weis JJ 1984; Molina H 1990), but not on immature B cells and plasma cells (Braun M 1998). $IRF4^+$ cells on the GC-T zone interface do not express CD21 (Fig 4.18 E). Taken together these data suggests that $IRF4^+$ cells on the interface have completely lost GC B cell identity.

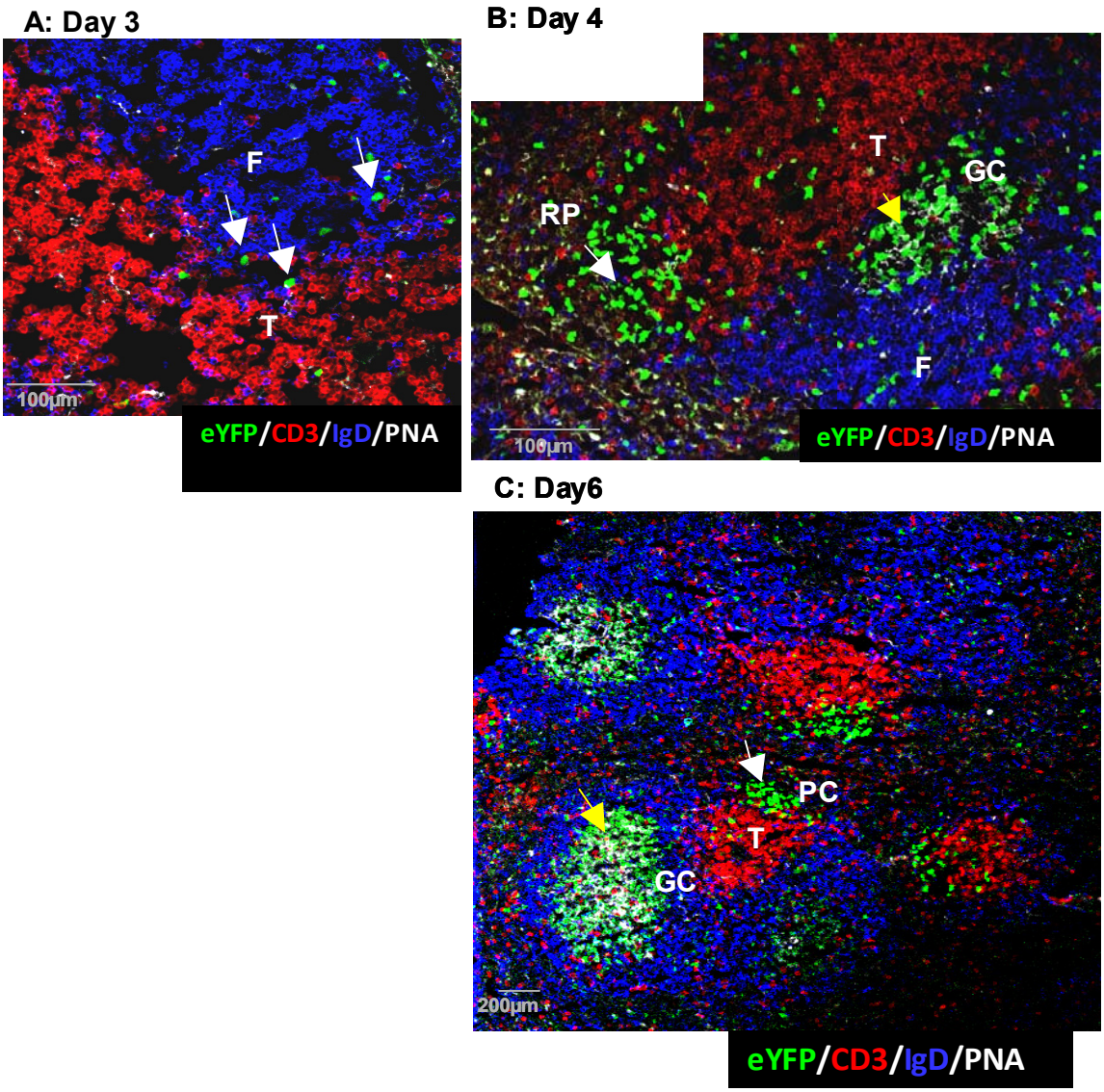

Figure 4.16: Appearance of activated B cells in Cg1-Cre x ROSA eYFP mouse spleen tissue after immunization

Immunofluorescent analysis of spleen section from day3 (**A**), day4 (**B**), and day6 (**C**) after immunisation with SRBC. Day3, just a few activated B cells (eYFP+) at the follicle/T zone boundary; at day4, small eYFP+ GC and the extrafollicular foci appeared; at day6, big eYFP+ GC developed, and more high level eYFP expression plasma cells produced. F: Follicle, T: T zone, GC: Germinal centre, RP: Red pulp. PC: Plasma cell.

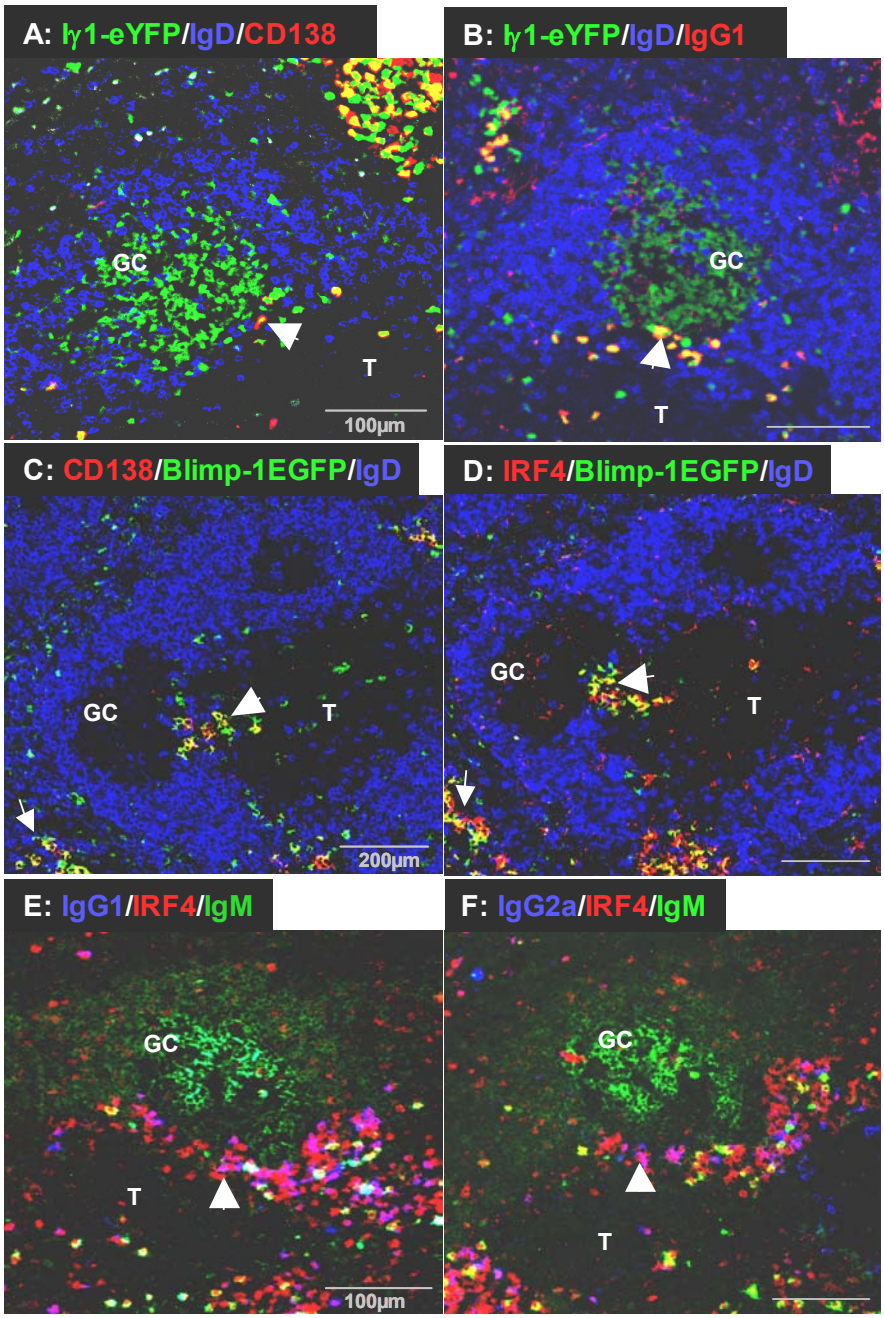

Figure 4.17: IRF4$^+$ cells at the GC-T zone interface express typical plasma cell markers

A. B: Iγ1-eYFP cells appear at the GC-T zone interface from Cg1-Cre x ROSA eYFP mouse at d6 after immunization with SRBC.They are CD138$^+$ and IgG1 switched. **C. D**: Blimp-1EGFP positive cells appear at the GC-T zone interface from Blimp-1EGCP mice at d6. They express both CD138 and IRF4. **E.F**: Some IRF4$^+$ cells at the GC-T zone interface already express switched IgG antibody at d5 after immunization with SRBC in C57BL6 mice. GC: Germinal centre, T: T zone.

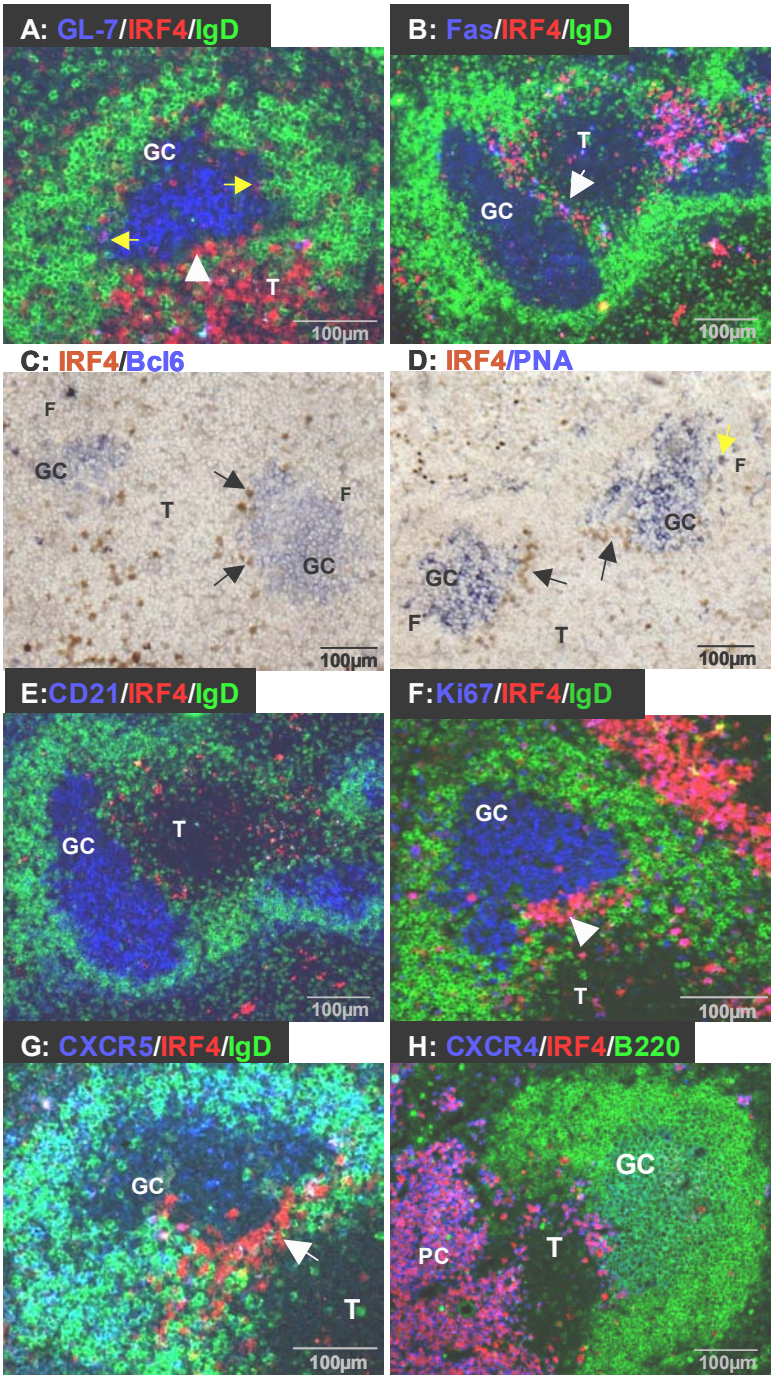

Figure 4.18: IRF4$^+$ cells at the GC-T zone interface already lose GC identities
C57BL/6 mice were immunized with SRBC. At d5, IRF4+ cells at the GC-T zone interface, already downregulate typical GC markers, such as: **A**: GL-7, **B**: Fas, **C**: Bcl6, **D**: PNA, and **E**: complement receptor CD21. They are Ki67+ (**F**), still doing proliferation. The downregulation of CXCR5 (**G**) and high expression of CXCR4 (**H**) guides IRF4+ cells to move out from the DZ-T zone interface, into red pulps. GC: Germinal centre, T: T zone. PC: Plasma cell.

The nuclear antigen Ki67 is a proliferation marker expressed only in cycling cells (during all phases of cell cycle: G_1, S, G_2 and mitosis), but not in resting cells (G_0) (Scholzen T 2000). Immunohistology shows that most $IRF4^+$ cells on the GC-T zone interface were undergoing proliferation ($Ki67^+$), like plasmablasts in the red pulp (Fig 4.18F), which indicates that these cells were still proliferating blasts, not yet differentiated to mature plasma cells.

Expression of CXCR5 directs B cells to home into the follicles (Forster R 1996). When B cells differentiate into plasma cells, sometime they downregulate CXCR5 and CCR7, and upregulate CXCR4, which recruits cells to localize in the red pulp and subsequently accumulate in the bone marrow (Hargreaves DC 2001). IRF4 cells on the GC-T zone interface were seen to completely downregulate CXCR5, and highly express CXCR4 (Fig 4.18 G H). Additionally, these cells also were observed to express only low levels of the B cell marker B220, which is typical for plasmablasts.

The BP-3 alloantigen is expressed on early B lineage, immature thymocytes, circulating neutrophils, peritoneal macrophages, and on subpopulation of stromal cells in the second lymphoid organs (McNagny KM 1988; McNagny KM 1991; Kaisho T 1994). Recent research suggests the BP-3 expression in lymphoid organs may be involved in leukocyte-stromal interactions which support the development, migration and responses of leukocytes (Ngo VN 1999). From the fluorescent staining, BP-3 expresses on the stromal cells in the white pulp, particularly on FDC areas (Fig 4.19 A). Interestingly, IRF4 cells appearing at GC-T zone interface are closely associated with BP-3 expressing stromal cells. This indicates that these stromal cells would be candidates for accessory cells providing signals to IRF4 expressing cells at the GC–T zone interface.

$CD3^-CD4^+$ LTi cells play an important role for organising the development and perhaps the maintenance of secondary lymphoid organs by the lymphotoxin-lymphotoxin receptor interaction (Mebius 2003). Studies from Prof. Lane group have suggested that adult LTi cells can mediate survival of T_{FH} cells and memory T cells, and maintain the affinity maturation in GCs and memory antibody response by the TNF family ligand OX40L and CD30L (Lane PJ 2005; Kim MY 2007; Withers DR 2007). Recently, a role for CD30 and OX40 from LTi cell was proposed for B cell activation in T independent responses (Kerry Perks, PhD thesis in preparation, UoB). Beside, localising in B follicles and the central T zone in spleen, LTi cells also sit at the interface of the B and T cell area, associated with the BP-3 positive stromal population (Fig 4.19 B C). Lti cell are identified as $CD3^-CD11C^-CD4^+IL\text{-}7R\alpha^+$ (Kim MY 2003). LTi cells close localisation with $IRF4^+$ cells in the GC-T zone interface indicates that LTi cells may be involved in the development and response of B cells, possibly through OX40/OX40L and CD30/30L interaction.

The summary table 4.1 presents all markers used to characterize the expression of IRF4 cells at the GC-T zone interface. In summary, $IRF4^+$ cells in this area are spatially very closely associated with the dark zone. As with most other GC B cells, they have undergone induction of IgG1 switching and have the same specificity as adjacent GCs. At the same time, these cells have already lost all GC B cell markers studied: GL-7, Fas, Bcl6, PNA, and complement receptor CD21, and have upregulated plasma cell markers, such as CD138, Blimp-1, and NP-specific Ig. They are still proliferating which identifies them as plasmablasts. High expression of CXCR4 and loss of CXCR5 recruits these $IRF4^+$ cells to the outer T zone and then to the red pulp. Therefore, $IRF4^+$ cells at the GC-T zone interface are early plasmablasts, exiting from the associated GC.

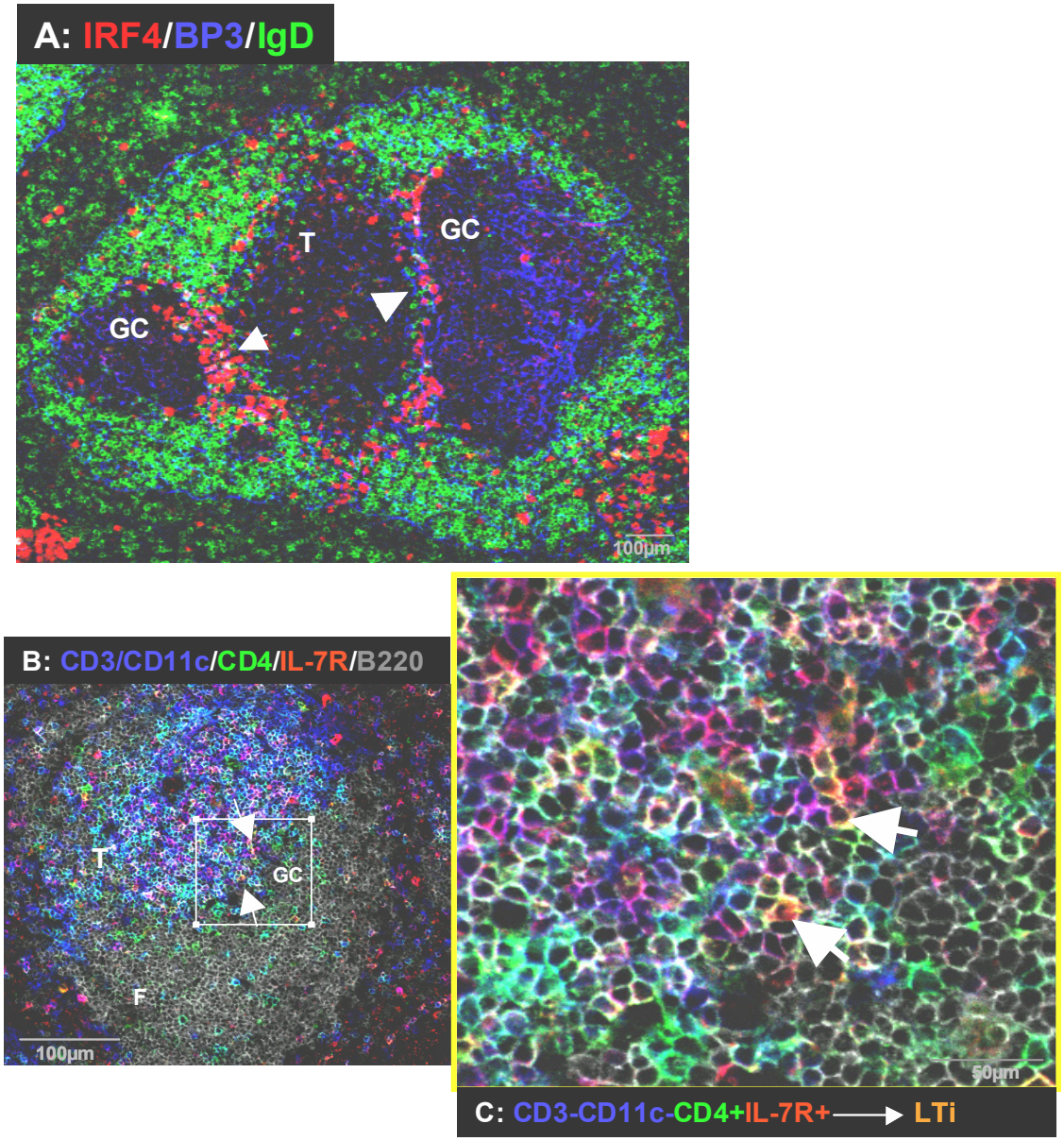

Figure 4.19: IRF4^{+} Cell associated with stromal cells and LTi cells
C57BL/6 mice were received SRBC. At d5, high expression of IRF4 cells appear at the GC-T zone interface, associated with BP-3^{+} stromal cells (**A**). (**B**) Adult LTi cells also localize in the follicle-T zone boundary. GC: Germinal center, T: T zone, F: Follicle. LTi cell:CD3-CD11C-CD4+IL-7R+

Marker Name	Expression of IRF4 cells on GC-T zone interface
NP-binding	Up-regulated
CD138	Positive
Blimp-1	Positive
IgG switched	IgG1 switched for Th2 antigen response
GL-7	Negative
PNA	IRF4 cells on border is neg, some cells are PNA+ in LZ
Fas	Some cells are Fas+, like plasma cells in the EF
Bcl-6	Negative
CD21	Negative
Ki67	Positive
CXCR5	Negative
CXCR4	Positive, like plasma cells in the EF
BP-3	Cells associate with BP-3^{+} stromal cells

Table 4.1: Expression of IRF4^{+} cells on the GC-T zone interface
Characterization the expression of IRF4^{+} cell seen at the GC-T zone interface at d5 after immunization with SRBC in C57BL/6 mice. These cells upregulate plasma cell markers, and lose GC identities at the same time. LZ: light zone, EF: Extrafollicular Foci.

4.2.4 Appearance of IRF4$^+$ cells in the follicle –T zone interface is T dependent

SRBC are complex and large antigens with repetitive carbohydrate epitopes. To test whether the differentiation of IRF4-expressing cells at the follicle–T zone interface is dependent on the presence of signals from T cells, CD3ε KO mice (Wang N 1998) were immunized with SRBC. Peanut agglutinin (PNA) as a GC B cell marker (Jacob J 1991) to stain spleen sections at day5 after immunization shows that no GCs were detected on spleen sections (Fig 4.20A). Some plasma cells that expressing IRF4 (Fig 4.20B) and CD138 (Fig 4.20C) were produced in the red pulp, showing that SRBC induce some T independent B cell activation. SRBC also induces some IgG switching in the extrafollicular response (Fig 4.20D), which was mainly switching to IgG_{2b} (Fig 4.20E), and a few cells are IgG3 (Fig 4.20F). This type of switching is typical for a TI-1 antigen. Importantly, SRBC do not induce GC development, and do not induce the appearance of significant numbers of plasmablasts at the follicular–T zone interface 5 days after immunization.

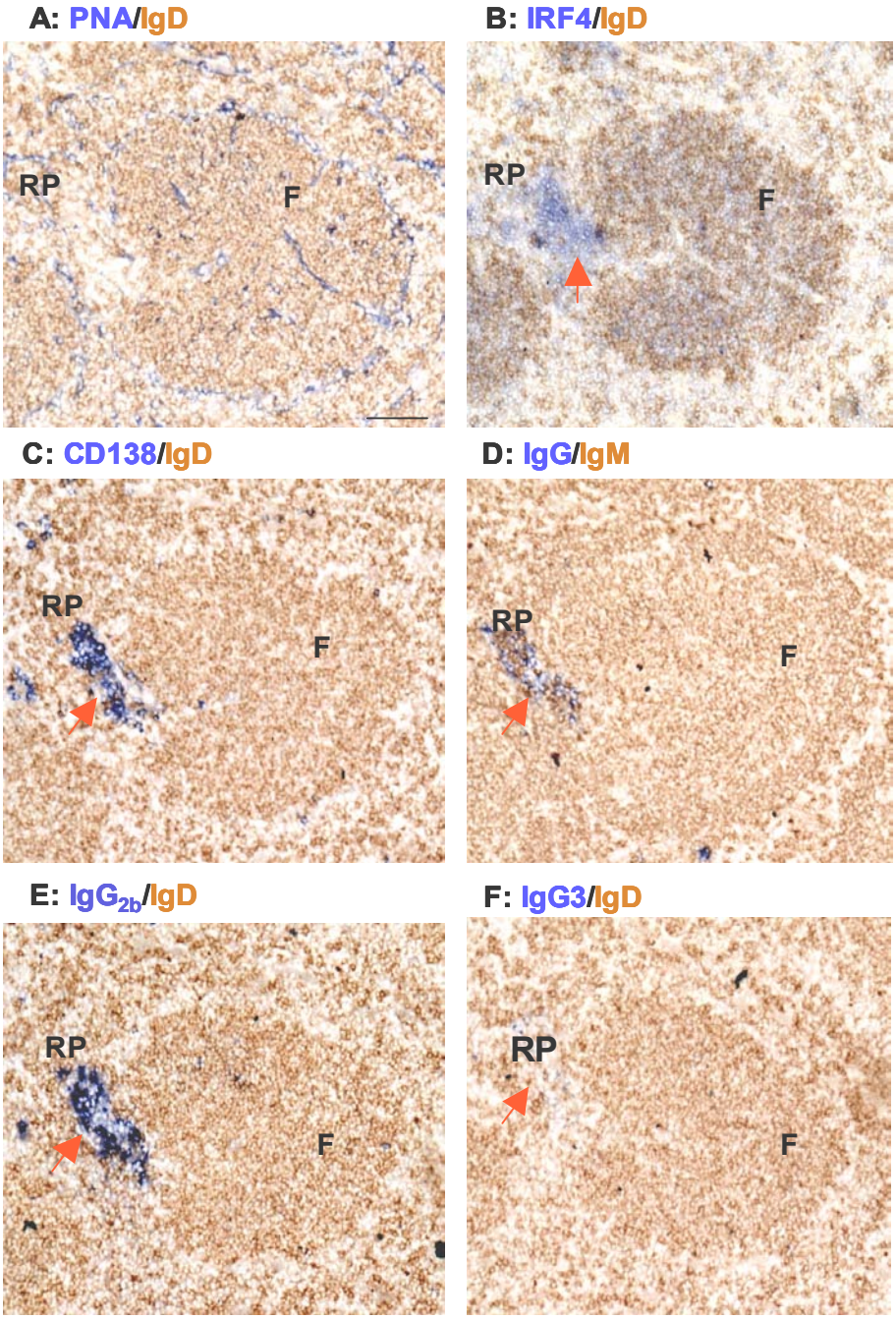

Figure 4.20: The response for SRBC in CD3ε KO mice

CD3ε KO mice were immunized with SRBC. At d5, no GC was detected in spleen section (**A**), and less plasma cells produced in the red pulp. (**B.C**). Mainly these plasma cells in the red pulp are IgG2b switched (**D.E.F**). F: Follicle, RP: red pulp. Scale bar:100 µm

4.2.5 The effects of absence of cytokines or costimulatory signals for the generation of IRF4 cells on the GC-T zone interface.

Next we sought to identify signals that may induce differentiation of blasts at the GC–T zone interface. A range of cytokines and costimulatory signals produced by T cells, or other cells, which could affect GC B cell differentiation and induce differentiation of plasma cells, were tested by immunizing mice with targeted deletions for cytokines (IL-4, IL6, IL13, IL10, IL21) or constimulatory signals (CD28, OX40, CD30).

IL-4, a key Th2 cytokine, induces differentiation of naïve CD4 T cells into Th2 cells, and the generation of Th2-derived cytokines (Chatelain R 1992; Kopf M 1994). For B cells, it can stimulate the proliferation of activated B cells, upregulate the production of MHC class II, and induce class switching to IgE (Hinton HJ 1999). IL-13, which is closely related to IL-4, also affects Th2 development, upregulates MHC class II expression and induces the secretion of IgE in activated B cells (McKenzie GJ 1998; McKenzie GJ 1999). Compared to IL-4, IL-13 is a more central mediator of the physiological changed induced by allergy in many tissues (e.g. Asthma) (Wills-Karp M 1999; Wynn 2003). We selected pure IL-$4^{-/-}$ BALB/c (Kuhn R 1991; Kopf M 1993) and IL-$13^{-/-}$ BALB/c mice (McKenzie GJ 1998a) to detect whether IL-4 or IL-13 could have some role for the generation of early plasmablasts exiting from the GC-T zone interface. However no significant difference in GC size and the production of $IRF4^{+}$ cell at the GC-T zone interface were detected in single IL-4 or IL-13 deficient mice by comparing to WT at day5 and 7 after immunization (data not shown). IL-4 and IL-13 have overlapping functions for initiating Th2 cell-driven responses. In the absence of one cytokine the other may compensate, resulting in Th2 cell differentiation, B cell activation and development (Cunningham AF 2002).

IL-4 and IL-13 play key roles in the Th2 immunity, regulate the activation of DCs, and they are functionally linked by their requirement for IL-4Rα for signalling (Nelms K 1999; Webb DC 2007). IL-4Rα$^{-/-}$ mice (BALB/c background) are impaired in IL-4 and IL-13 functions (Mohrs M 1999). This helps understand the severely impaired GC formation at d5 after SRBC injection, probably due to the impaired functions of CD4^{+} Th2 cells for the development of B cells, so IRF4+ cells on the B-T interface can't be analysed.

CD28, a B7 receptor expressed on T cells, can provide co-stimulatory signals, and is required for T cell activation (June 1990). CD4 T cells are essential for the GC formation in the TD response (Han 1995b). CD28$^{-/-}$Balb/c mice have a severely reduced capacity to form GC and a diminished humoral immune response (Shahinian A 1993; Reiter R 2002). As expected, we found that GCs were undetectable in CD28$^{-/-}$BALB/c mice after SRBC injection (data not show). There also was no appearance of IRF-4^{+} cells on the T zone–follicle interface 5 days after immunization, confirming that this cell population is GC derived.

4.2.5.1 The effect of CD30 / OX40 for the IRF4 cells on GC-T zone interface

CD30 and OX40 (CD134) are members of the tumour necrosis factor receptor (TNFR) family, and have costimulatory effects on T and B cells (Watts 2005; Kennedy MK 2006; Sabbagh L 2007). CD30 was originally identified in Hodgkin's lymphoma and Reed-Sternberg cells (Stein H 1982b). CD30 is expressed on activated T, B and NK cells (Horie R 1998). Its ligand (CD30L) is mainly expressed on resting B cells, activated T and B cells, antigen-presenting cells and a subset of accessory cells (such as LTi cells) (Kim MY 2003; Watts 2005). Recent research supports that CD30 may have important roles in autoimmune diseases, such as asthma (Polte T 2006; Polte T 2009) and diabetes (Chakrabarty S 2003). Additionally, CD30 and CD30L engagement downreguates isotype

switching in normal human B cells whose differentiation is mediated by CD40L (Cerutti A 1998; Cerutti A 2000; Cerutti A 2001). More interestingly, CD30 signal was identified to have an important function in effective B/T segregation (Bekiaris V 2007).

OX40 is a secondary costimulatory molecule, mainly expressed on activated T cells, and has critical functions for mediating T cell activation (Croft 2003; Croft M 2009). OX40L is expressed on a number of cell types such as activated B and T cell, CD40L activated DCs (Fillatreau 2003), and accessory cells (e.g. Lti cells) (Kim MY 2003). OX40/OX40L has a critical role in maintaining an immune response beyond the first few days, and consequently governing the memory response due to determine the memory T cell pool (Croft 2003; Croft M 2009; Withers DR 2009). Many studies have reported that OX40-OX40L interaction could regulate autoimmune and inflammatory reactions (Watts 2005).

Recently Lane's group identified $CD4^{+}CD3^{-}CD11c^{-}$ LTi cells which costimulate primed CD4 T cells through OX40 and CD30 at the B:T interface and in follicles (Kim MY 2003). OX40 and CD30 signals act together to control the development of memory T cells to provide help to maintain GC development, and secondary antibody responses (Gaspal FM 2005; Sabbagh L 2007; Lane PJ 2008). A recent study showed that LTi derived CD30 has an inhibitory effect, while OX40 has stimulatory activity in TI-I/TI-II B cell responses (K. Perks, thesis in preparation). OX40 and CD30 double deficient mice have been produced by crossing OX40 and CD30 single KO mice, described before (Amakawa R 1996; Pippig SD 1999) (Gaspal FM 2005), mice kindly received from Prof. Lane, Birmingham UK)

Germinal centres were observed in all mice on day 5 and 7, illustrating that signals absent in CD30KO, OX40KO, and dKO mice were not required for the initiation of GC

formation. However, there is significant bigger GC development in OX40KO and dKO ($p<0.01$). This effect was only observed on day5, and disappeared on day 7 (Fig 4.21 A B C D E).

More $IRF4^+$ cells were observed at the GC-T zone interface in CD30KO mice ($p<0.01$), and $IRF4^+$ plasma cells in red pulps were far more numerous in KO mice (Fig 4.21B, F). As a previous study showed no notable effect on B cell responses in CD30KO mice (Kennedy MK 2006), we confirmed this phenotype by fluorescent staining to verify that IRF4 cells on the GC-T zone interface in KO mice already show a plasma cell phenotype: CD138, IgG1 antibody, and chemokine CXCR4 (Fig 4.22).

OX40 deficiency resulted in significantly less $IRF4^+$ cells at the GC-T zone interface ($p<0.01$) (Fig 4.21F), and also much lower numbers of $IRF4^+$ plasma cells in red pulps. Therefore, signals from OX40 and CD30, both of which may be derived from LTi, seem to have opposite effects not only on TI-I/TI-II B cell activation, but also on the appearance of early GC-derived plasmablasts.

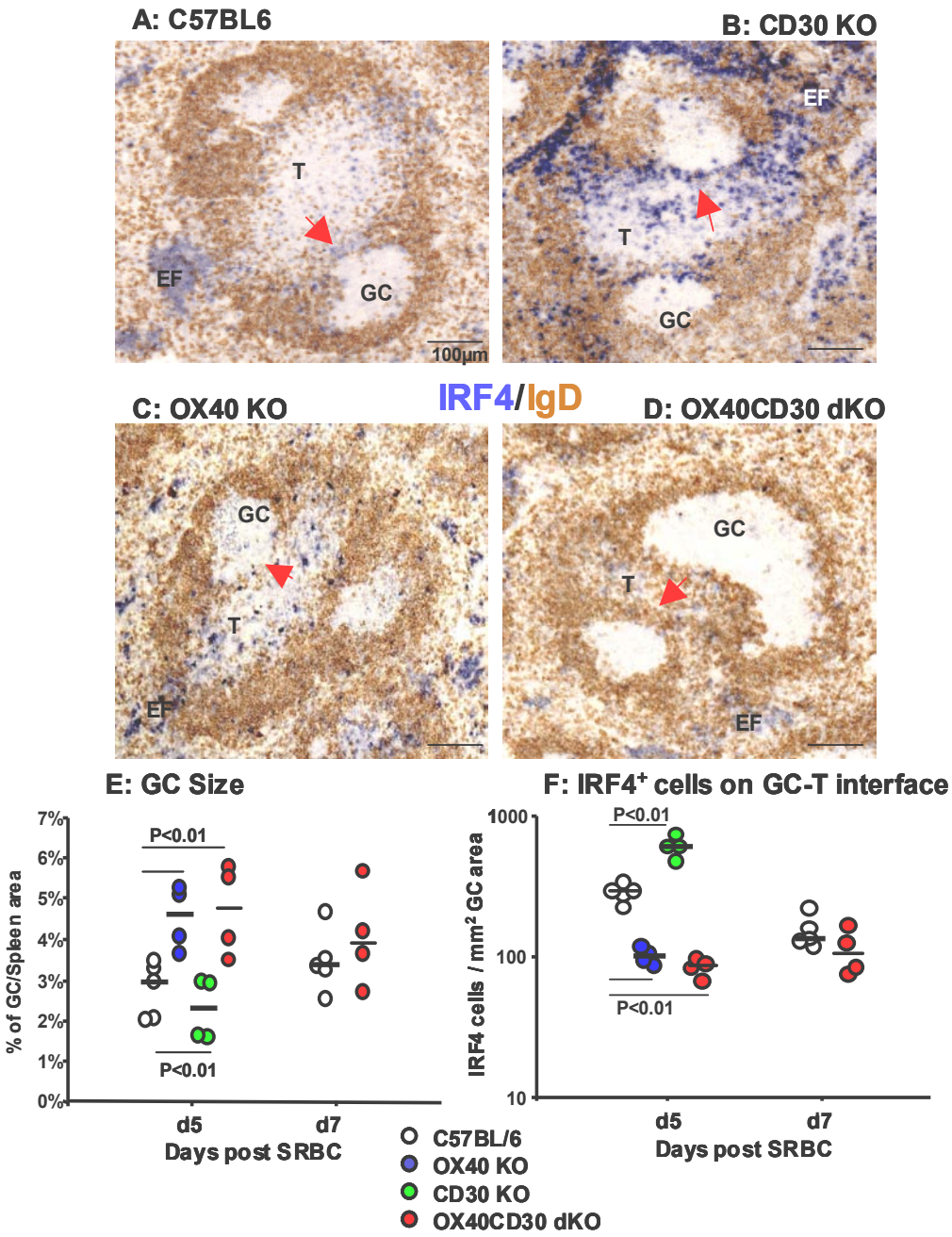

Figure 4.21: The effect of OX40, CD30 single and double deficiency for production of IRF4$^+$ cells at the GC-T zone interface

OX40, CD30 single deficient and double deficient mice, C57BL/6 as control were immunized with SRBC. Representative spleen section from control C57BL/6 (**A**), CD30 single KO (**B**), OX40 single KO (**C**), CD30OX40 double KO (**D**), respectively, was stained to reveal the number of IRF4$^+$ cells at the GC-T zone interface at day 5. **E**: the quantification result of GC size per spleen section from C57BL/6, CD30$^{-/-}$, OX40$^{-/-}$, and OX40CD30 double KO mice, **F**: the quantification of IRF4$^+$ cells at the GC-T zone interface on sections from different species of mice. Cell was assessed per mm^2 GC area. Each point represents one animal. P<0.05 according to the Wilcoxon Mann-Whitney U test. GC: Germinal centre. T: T zone.

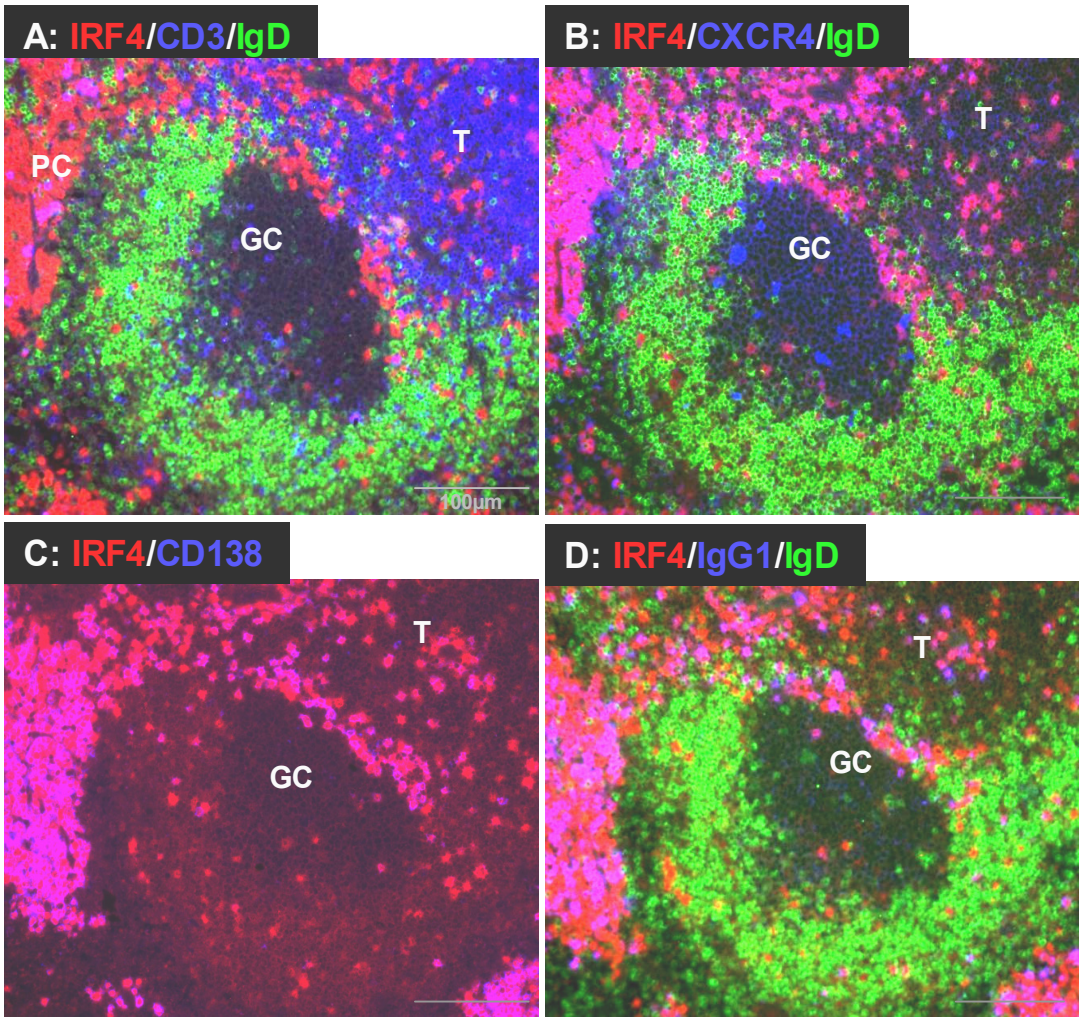

Figure 4.22: IRF4^{+} cells at the GC-T zone interface in CD30KO mice
Representative spleen section from CD30 single deficiency mice at d5 after immunization with SRBC, was stained with (**A**) CD3/IRF4/IgD, (**B**) IRF4/CXCR4/IgD, (**C**) CD138/IgD, (**D**): IRF4/IgG1/IgD. IRF4^{+} cells already express low CD3, high level of CXCR4, CD138 and IgG1 switched Ig. GC: Germinal centre, T: T zone; PC: Plasma cell

4.2.5.2 The effect of IL-6/IL-10 for the IRF4 cells on GC-T zone interface

IL-6 is an immunomodulatory cytokine with a wide range of biological activities. For B cell, IL-6 can act as a growth factor, and regulate plasma cell differentiation and Ig secretion (Van Snick 1990; Tanner JE 1992; Kopf M 1994). Furthermore, it has a profound effect on T cells, for example stimulating T cells differentiate into cytotoxic T cells, and more importantly regulating CD4 T cell proliferation, survival and differentiation (Dienz O 2008).

IL-10 is an important immunoregulatory cytokine that suppresses functions of Th1 cell, macrophages, DCs and NK cells (Howard M 1992; Moore KW 2001; Mocellin S 2003). It is mainly produced by subsets of T-cells e.g. natural Treg, effector CD4 T cells, with diverse roles in contribution in T cell-mediated immune response (Maynard CL 2008). In addition, IL-10 augments the proliferation and differentiation of B cells (Moore KW 1993). To investigate the role of IL-6 or IL-10 in the GC response, IL-6 and IL-10 single KO mice were immunized with SRBC, the KO mice were described before ((Kopf M 1994) and (Kühn R 1993), the mice kindly abtained from Dr. A. Cunningham Birmingham UK).

Germinal centre development in the absence of IL-6 was delayed, with no or only small GC observed at day5 after injection. GC formed at day7 (Fig 4.23 A B), but only half of mice had GC comparable in size to WT mice. This confirms that IL-6 regulates the initiation of GC formation (Kopf M 1998). The number of IRF4 cells seen at the GC-T zone interface was much lower in KO mice at day5 and 7 ($p<0.01$ Fig 4.23F).

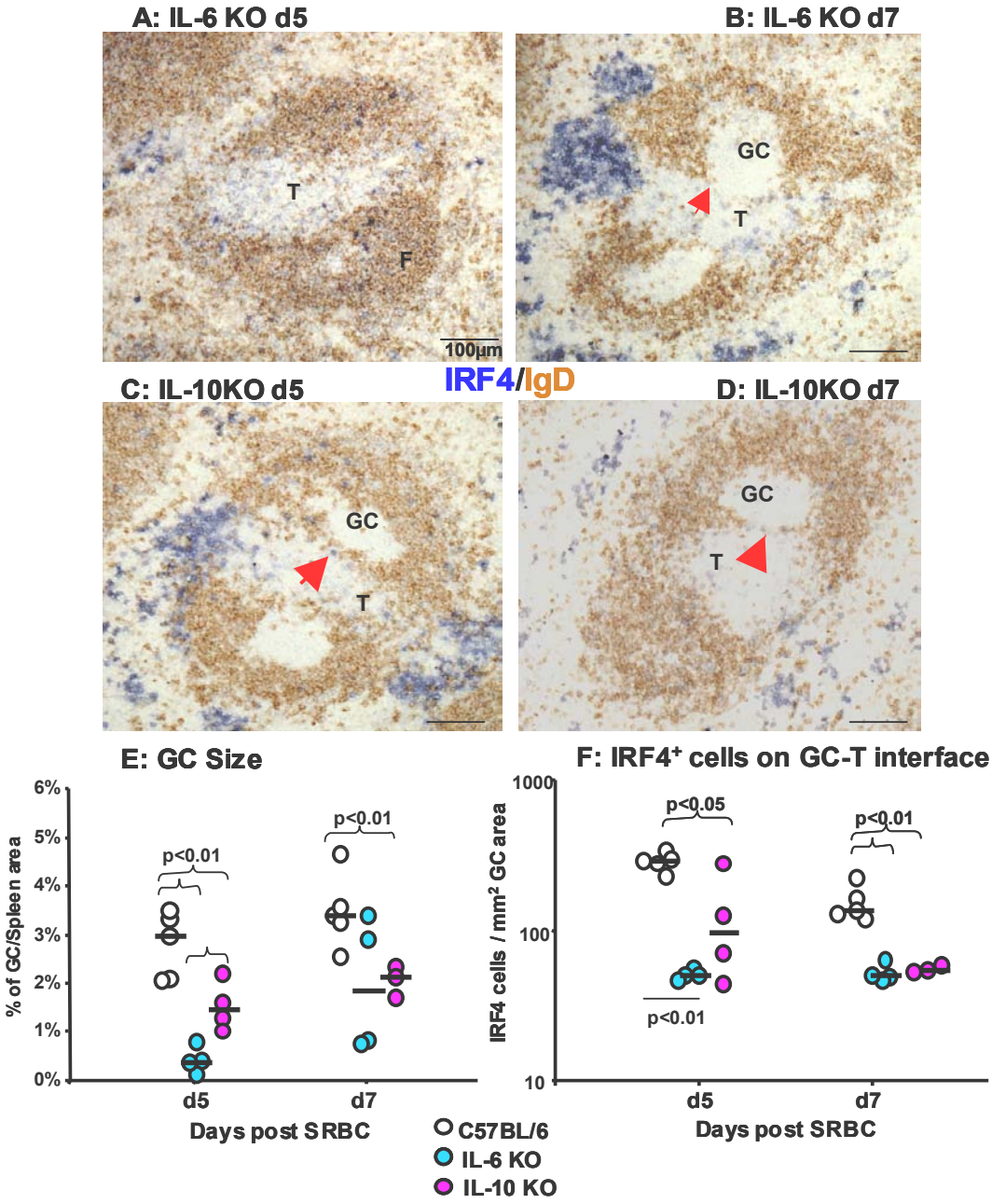

Figure 4.23: The effect of IL-6 /IL-10 deficiency for production of IRF4$^+$ cells at the GC-T zone interface

IL-6-/-, IL-10-/- mice, C57BL/6 as control were immunized with SRBC. Representative spleen sections from IL-6-/- at day5 (**A**) and day7 (**B**), and from IL-10-/- at d5 (**C**) and d7 (**D**) were stained to reveal the frequency of IRF4+ cells at the GC-T zone interface. **E**: the quantification result of GC size per spleen section from C57BL6, IL-6-/-, IL-10-/- mice. **F**: IRF4+ cells at the GC-T zone interface were quantified on sections from different mice types. Cell was assessed per mm2 GC area. Each point represents one animal. P<0.05 according to the Wilcoxon Mann-Whitney U test. GC: Germinal centre. T: T zone. In

IL-10 KO mice, GC developed with normal kinetic, but the GCs were significantly smaller compared to WT (Fig4.23 C D E). This result suggests that IL-10 is not as important a factor for the initiation of GC formulation as IL-6, but may maintain GC development. $IRF4^+$ plasmablasts at the GC-T zone interface were significantly reduced compared to WT ($p<0.01$), similar to IL-6KO (Fig 4.23 F). IL-10 is essential for proliferation of B cells and differentiation of GC B cells into plasma cells secreting IgG and IgA (Maliszewski CR 1993; Choe J 1998). Therefore IL-10 deficiency may impair GC and plasma cell development.

4.2.5.3 The effect of IL-21 for the IRF4 cells on GC-T zone border

IL-21, secreted by T_{FH} cells, is associated with the growth and differentiation B and T cells through IL-21R (Ettinger R 2008). *In vitro*, IL-21 can increase both Blimp-1 and Bcl6 on B cells (Ozaki K 2004; Arguni E 2006). Recent research supports that IL-21 has a role in the development of T_{FH} cells, and may augment the formation of T_H-17 cells, which secrete IL-21 and promote GC formation (Korn T 2007; Zhou L 2007). Latest research from Zotos (2010) utilising mice lacking IL-21 or IL-21R (KO mice described in (Ozaki K 2002)) further suggests that IL-21 is required for plasma cell formation through IL-21R on B cells. Spleen tissue from IL-21 and IL-21R deficient mice was assessed for GC formation and the production of $IRF4^+$ cells at the GC-T zone interface after immunization with alum precipitated NP-KLH (Tissue kindly provided by Dr. D. Tarlinton Australia).

Germinal centre and plasma cell formation were determined by immunohistological staining at day7 after immunization. IL-21/IL-21R deficient mice have smaller GCs, but higher number of B blasts occurring in follicles (Fig. 4.24 A-C, F, and (Zotos D 2010)). This indicates that IL-21 may have a role for migration of antigen-specific B cells before GCs form.

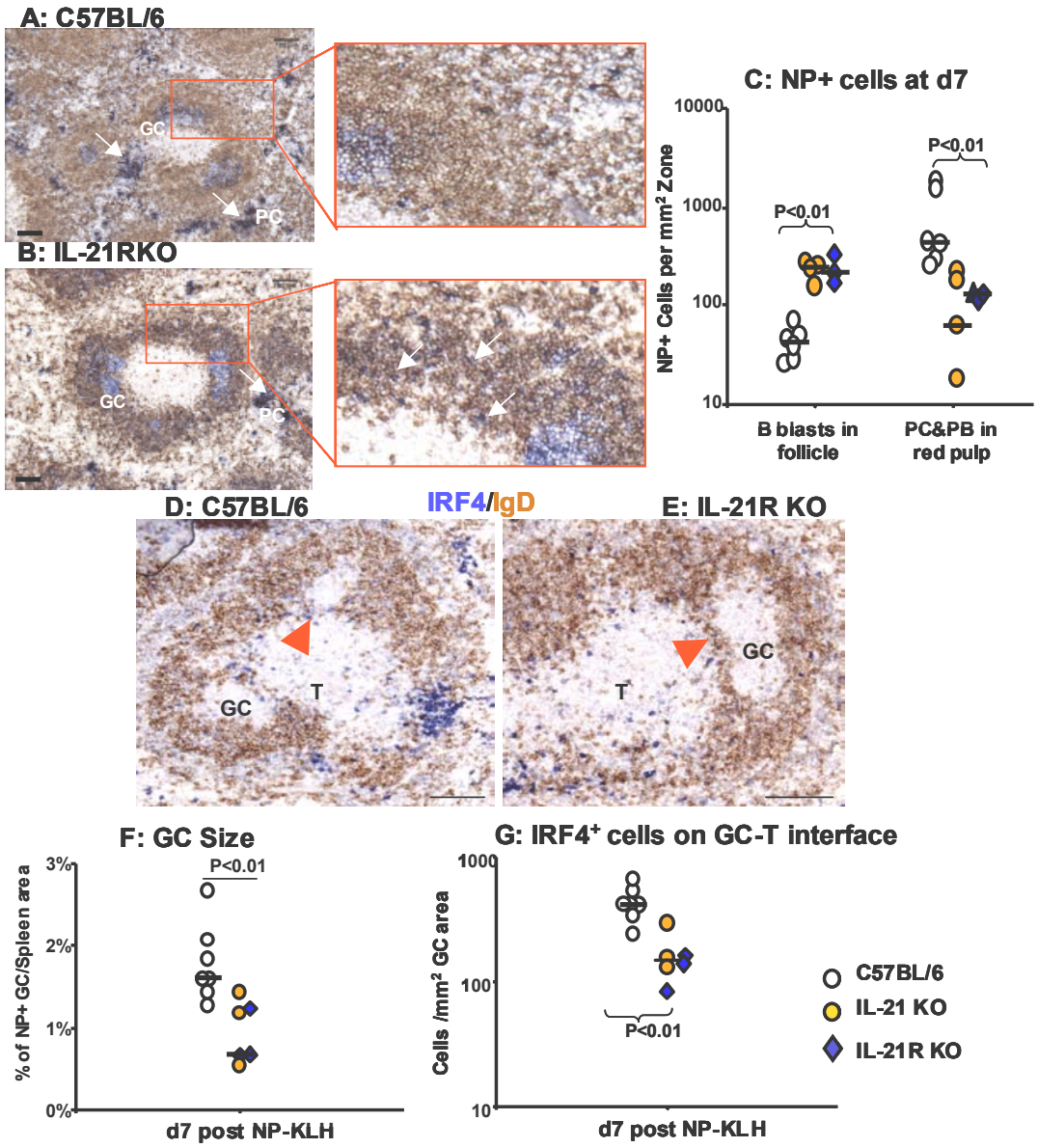

Figure 4.24: The effect of IL-21/IL-21R deficiency for production of IRF4$^+$ cells at the GC-T zone interface

IL-21-/- and IL-21R-/- mice, C57BL/6 as control were *i.p.* immunized with NP-KLH in alum precipitated. Representative spleen section histological staining reveals the localization of NP-reactive cells (blue) in control (**A**) and IL-21R$^{-/-}$ (**B**) mice. NP$^+$ cells in EF and follicles are shown by arrows. **C**: Quantification of NP specific plasmablasts (PB) and plasma cells (PC) in the red pulps and NP specific B blasts in follicles from control C57BL/6, IL-21-/- and IL-21R-/- mice at day7 (A-F data published in Zotos 2010). Histological staining shows the distribution of IRF4 cells on control (**D**) and IL-21R-/- (**E**) at day7 . **F**: Quantification of GC size per spleen section from C57BL6, IL-21-/- and IL-21R-/- mice, **G**: IRF4$^+$ cells at the GC-T zone interface were quantified. Cell was assessed per mm2 GC area. Each point represents one animal. P<0.01 according to the Wilcoxon Mann-Whitney U test. GC: Germinal centre. T: T zone. Scale bar: 100 µm

IRF4 staining showed less IRF4^{+} cells appearing at the GC-T zone interface in KO mice, even when the number of these cells was related to the GC area ($p<0.01$ Fig 4.24 G), consistent with the deficiency of NP specific plasma cells in red pulps ($p<0.01$ Fig 4.24 C). The results suggest that signals from T_{FH} cells have a role in the response and differentiation of GC–T zone interface plasmablasts.

4.2.5.4 The effect of IL-22 for the IRF4 cells on GC-T zone interface

IL-22, another member of IL-10 family, exerts an important mediator in regulate the inflammatory response (Dumoutier L 2000; Wolk K 2006). Its receptor (IL-22R) is a cell-surface complex composed of IL-10R2 and IL-22R1, which is mainly expressed on epithelial cells of organs in respiratory tracts and gut, and the skin, but not on immune cells such as B cells (Wolk K 2006; Jones BC 2008). Recent studies support that IL-22 could mediate early host defense against microbial pathogen, tissue repair, wound healing and the homeostasis of epithelia (Wolk K 2006; Zenewicz L 2007; Zheng Y 2008; Pickert G 2009). IL-22 is mainly produced by NK cells (NKp44^{+}), T_H-17 cells, and LTi-like cells in mice (Kreymborg K 2007; Colonna 2009; Takatori H 2009; Vivier E 2009). As IL-10 has a role for the production of GC-T zone interface plasmablasts, we tested this member of the IL-10 family for similar effects.

IL-22-/-BALB/c mice (Zheng Y 2008), kindly received from Prof. C. Buckley Birmingham UK) were immunized with SRBC as before. No difference in GC size and plasma cell numbers were observed between KO and WT mice (Fig 4.24 G). Interestingly, more T cells were seen in GCs of KO mice at day5 (Fig 4.25 A B). SRBC induce bigger GC in BALB/c mice compared to C57BL/6, and far less IRF4 cells localizing at the GC-T zone interface (data not shown). GC developed to normal sizes compare to wt controls (Fig 4.25 C D). Surprisingly, more IRF4 cells appeared at the GC-T zone interface in KO mice (Fig 4.25 E F).

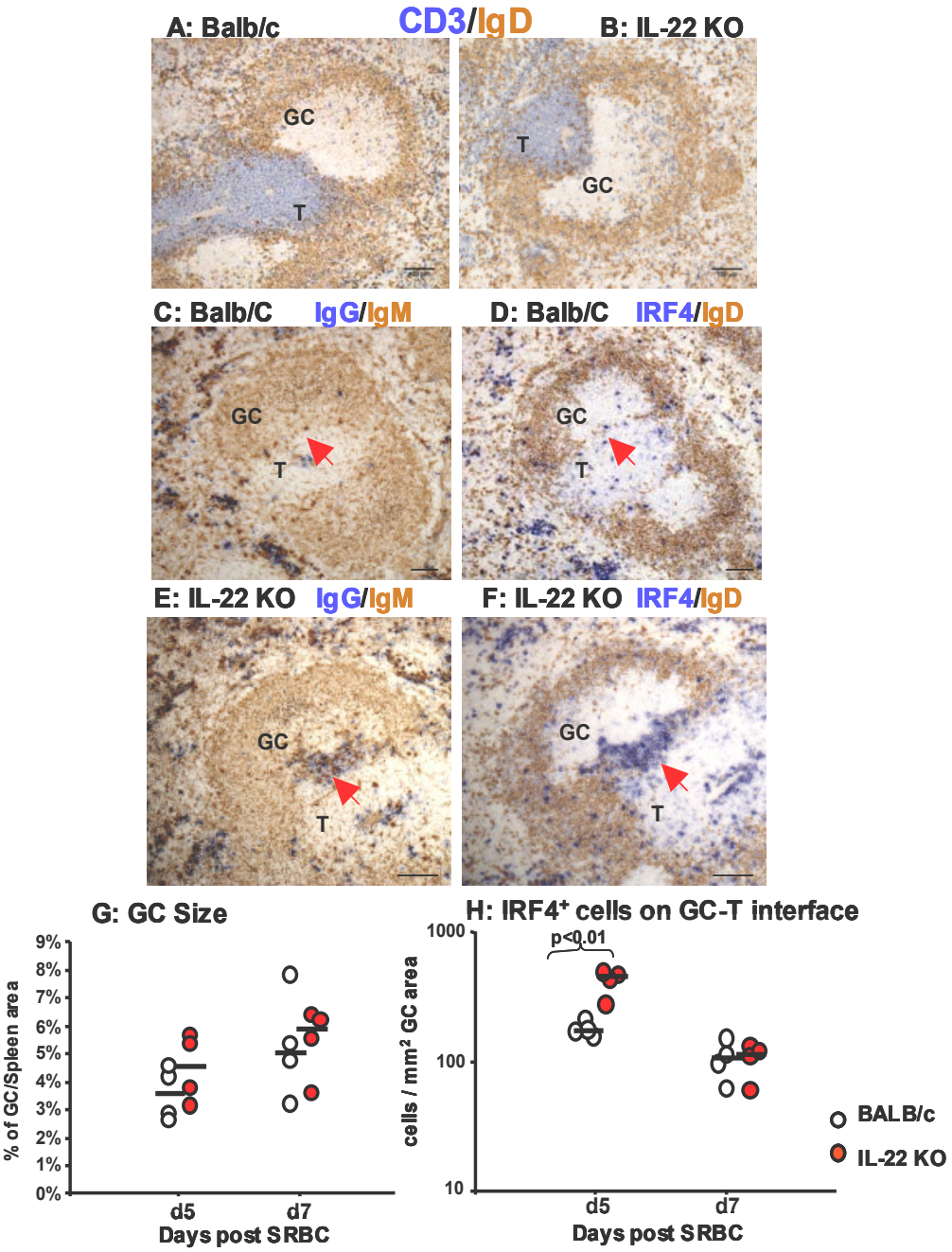

Figure 4.25: The effect of IL-22 deficiency for production of IRF4$^+$ cells on GC-T zone interface

IL-22-/- and BALB/c as control were immunized with SRBC. Representative spleen histological staining with CD3 reveals the frequency of T cells in control (**A**) and IL-22-/- (**B**) mice at day5. Staining with IgG and IRF4 at the serial sections is going to compare the IRF4+ cells at the GC-T zone interface between control (**C, D**) and IL-22-/- mice (**E.F**) at day5. **F**: Quantification of GC size per spleen section from Balb/c and IL-22-/- mice, **G**: IRF4+ cells at the GC-T zone interface were quantified. Cell was assessed per mm2 GC area. Each point represents one animal. P<0.01 according to the Wilcoxon Mann-Whitney U test. GC: Germinal centre. T: T zone.

Quantification showed a significant difference at day5 compare to wt mice ($p<0.01$), but this effect disappeared at day 7 (Fig 4.25 H). Direct effects of IL-22 on B cells so far have not been described. It is possible that deficiency of IL-22 might affect the differentiation of CD4 T cells in GCs, resulting in effects on selection of GC B cells, finally resulting in higher plasma cells output from GCs. The abnormal function of stromal cells or Lti cells due to IL-22 deficiency also might affect the early plasma cell development.

The table 4.2 summarizes the impact of all cytokines and costimulatory signals used so far for the generation of $IRF4^+$ plasmablasts localized at the GC-T zone interface.

Mouse stain	GC response	$IRF4^+$ cells on GC-T interface
IL-4	No difference with WT	No difference
IL-13	No difference with WT	No difference
IL-4Ra	Very small GC formed at d5	No $IRF4^+$ cells was detected at the B/T boundary
CD28	Very small GC formed or None	No $IRF4^+$ cells was detected at the B/T boundary
IL-6	Delay GC response to d7, small GC	Less $IRF4^+$ cells
CD30	Small or similar GC	More $IRF4^+$ cells
OX40	Bigger GC	Less $IRF4^+$ cells
OX40CD30	Bigger GC	Less $IRF4^+$ cells
IL-21	Similar GC	Less $IRF4^+$ cells
IL-10	Small GC	Less $IRF4^+$ cells
IL-22	More GC T cells	More $IRF4^+$ cells at early response (d5)

Table 4.2: Summary of the effect of Cytokines or co-stimulatory signals for $IRF4^+$ cells at the GC-T zone interface
Different cytokine or co-stimulatory signal deficient mice were used to detect which signal could be involved in the generation of the $IRF4^+$ plasmablasts at the GC-T zoon interface. GC: Germinal centre, WT: wild type mice.

4.2.5.5 Appearance of IRF4$^+$ cells in the TI primary response

To assess whether the presence of these IRF4$^+$ cells on the interface is dependent on TD signals, the appearance of IRF4$^+$ cells in TI GC response to NP-Ficoll on spleen tissue was studied. TI GCs do not have B-T cell interaction and B selection in GC, which is thought to be the reason for the sudden convolution of TI GC by mass apoptosis at day 6 after immunisation (Vinuesa 2000). This study was carried out via using QMxC57BL/6 mice carrying high frequencies of NP-specific cells (described in section 2.1.1 Vinuesa 2000). NP-specific activated B cells appeared in the outer T zone within hours after injection, and GC started to appear at day 3 (data not shown). Interestingly, IRF4 expressing cells were seen at the GC-T zone interface beginning from day3 (Fig 4.26 B), and these cells were NP-specific (Fig 4.26 A). By day4, large antigen-specific GC had formed, and high levels of NP expressing plasmablasts were seen at the GC-T zone interface (Fig 4.26 C), which were IRF4$^+$ (Fig 4.26 D). The quantification showed that the numbers of IRF4$^+$ cells at the GC–T zone interface were comparable to response to TD antigen (Fig 4.26 E). This result indicates that appearance of IRF4$^+$ plasmablasts at this location may be induced by signals or cytokines from stromal cells, DCs, or CD4$^+$CD3$^-$ LTi cells, which localise at the B/T interface and within follicles, and is not related to the B–T cell interaction.

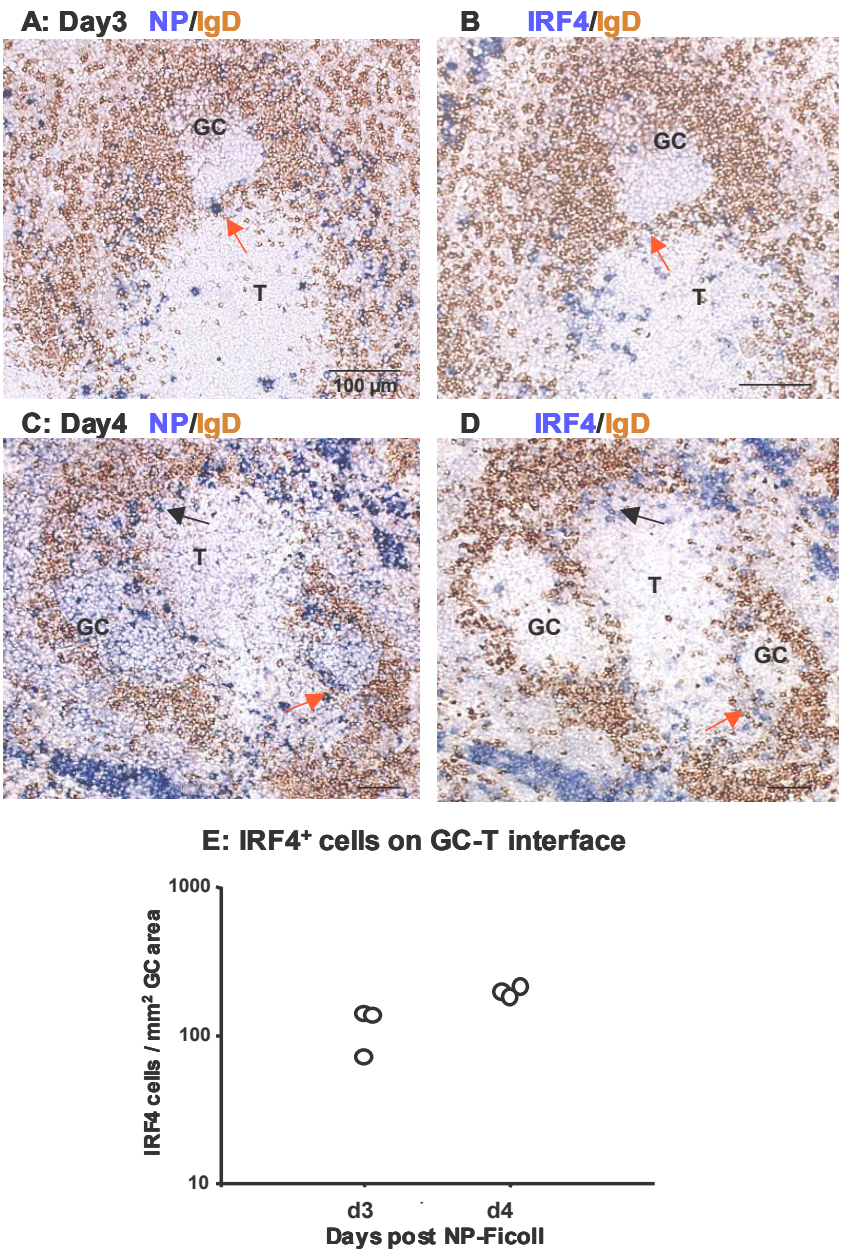

Figure 4.26: Appearance of IRF4$^+$ cells at the GC-T zone interface in the TI GC response

The appearance of IRF4+ cell at the GC-T zone interface on spleen sections was assessed at the time point after NP-Ficoll immunization in QMxC57BL/6, red arrow. The same spleen tissue stained with NP/IgD (**A**) and IRF4/IgD (**B**) at day 3 after immunization. The same tissue stained with NP/IgD (**C**) and IRF4/IgD (**D**) at day4. (**E**): Quantification of the density of IRF4+ cells at the GC-T zone interface at d3 and 4. Cell number was assessed by per mm2 GC area. Each point represents one mouse. GC: Germinal centre, T: T zone. Represent of two individual experiments.

4.2.6 Detecting presence of differentiation signals at the mRNA level by microdissection and RT-PCR

To look for the presence of chemokines and or cytokines that may influence the early plasmablasts differentiation or migration *in vivo*, mRNAs encoding these were assessed in different compartments of the spleen. Laser capture microdissection (LCM) was performed to cut out the different areas from snap-frozen spleen tissue. Spleen tissue from d5 after NP-CGG immunisation in carried primed C57BL/6 mice was used in this series of experiments, because this tissue contains the highest numbers of $IRF4^+$ cells in the GC–T zone interface. Serial tissue sections were mounted on membrane-slides and stained by cresyl violet to show the red pulp and white pulp areas. This staining avoids damage of RNA. Adjacent sections to the sections used for microdissection were mounted on normal multispot slides, which were stained for IRF4 and IgD to show the detailed anatomy of the white pulp and GC-T zone interfaces containing $IRF4^+$ cells. 10-20 similar areas were microdissected, and saved in the same tube to prepare RNA. 5-6 different white pulp areas were selected per spleen section (detailed methods in section 2.7). Gene expression was measured by real-time RT-PCR in multiplex with β_2 microglobulin (β_2m) specific primers as a uniformly expressed control gene.

Prior to testing other gene expression, CD3ε and CD19 were chosen as area positive controls. As expected, CD3ε is highly expressed in T zones, with less expression in GCs and the GC-T zone interfaces. The later contains a mix of B cells and T cells. The lowest expression is in plasma cell areas, follicles and red pulps which containing few T cells. High expression of CD19 was seen in GCs, follicles and red pulps, with intermediate levels in the GC-T zone interfaces, and low level in T zones and plasma cell areas (Fig 4.27 A).

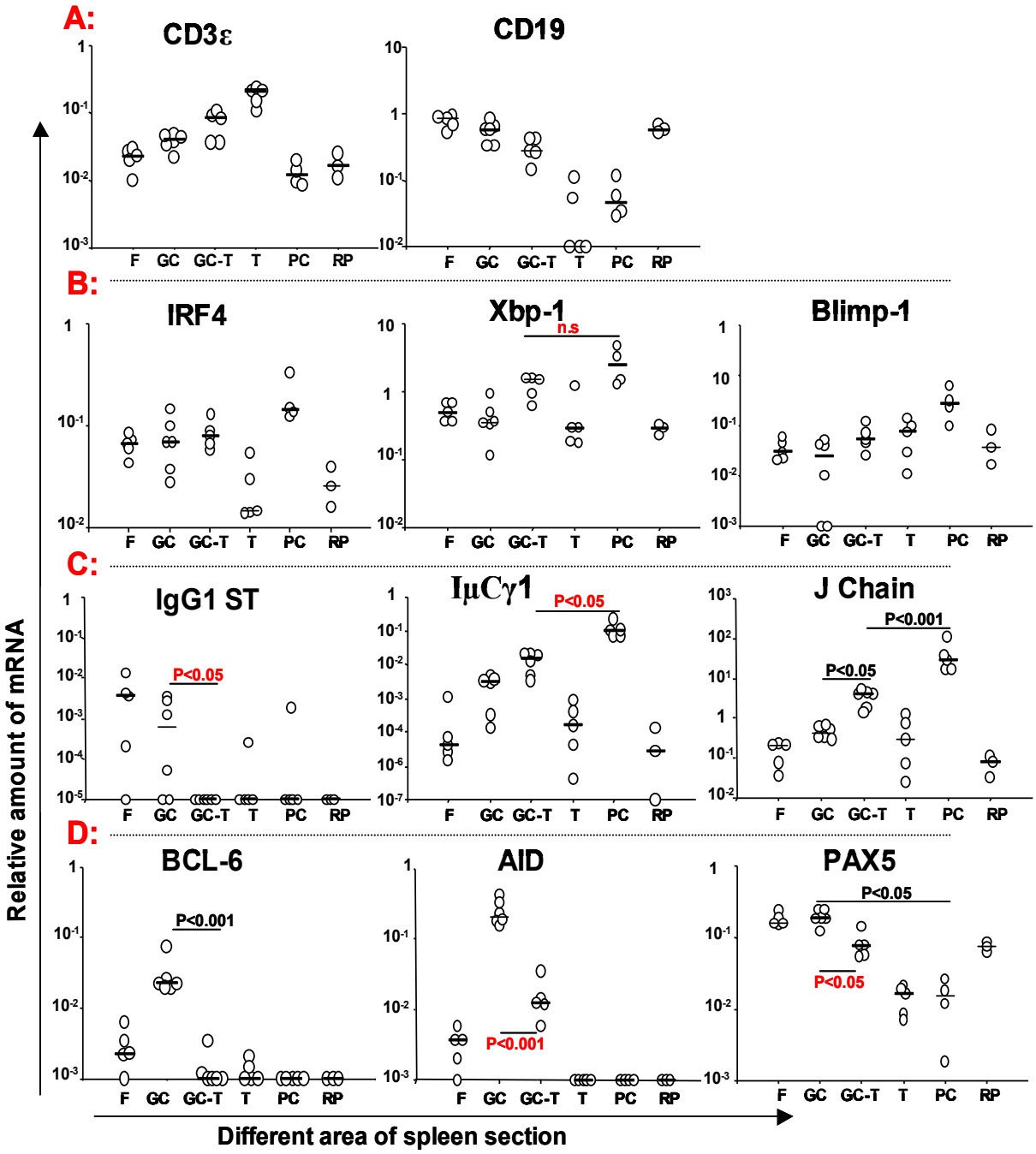

Figure 4.27: Plasmablast associated genes upregulated in IRF4$^+$ cells at the GC-T zone interface

A: Cells in at the GC-T zone interface are mixed T and B cells by accessing expression level of CD3ε and CD19. **B:** Genes associated with plasmablast differentiation are upregulated at the mRNA level in the GC-T zone interface; **C** : Gene associated with class switch transcript is low ($p<0.05$ between Gcand GC-T interface), and but associated with antibody production are upregulated in the same area. **D**: But genes associated with GC markers are downregulated ($p<0.001$ between GC and GC-T interface). Each spot represents one area of one spleen section. All values are relative to the house keep gene β_2-microglobulin. $p<0.05/p<0.001$ according to the Wilcoxon Mann-Whitney U test. This is representative of two individual experiment. GC-T: GC-T zone interface, GC: Germinal centre, T: T zone, PC: IRF4$^+$ plasma cell area, F: Follicle without GC. RP: red pulp with low/no IRF4$^+$ cells as control. n.s.: non-significance

4.2.6.1 The expression of plasmablast/plasma cell associated genes

As expected, IRF4 mRNA was mainly detected in cells at the GC-T zone interface and in plasma cells. IRF4 expression was also detected in GC cells, and follicle B cells. Low levels were seen in the T zone. The relatively high levels of IRF4 mRNA in GC cells may be derived from the small numbers of IRF4 positive centrocytes. As expected, other plasmablast and plasmablast precursor associated genes, such as the transcriptional regulator of the plasma cell secretory phenotype XBP1 (Shaffer AL. 2004), and Blimp1 (Shapiro-Shelef M 2003) are upregulated at the GC-T zone interface as well (Fig 4.27 B). XBP-1 is present in lower levels in GC, follicular and red pulp areas, and at much higher level in plasma cells. Blimp-1 is expressed in the T zone and follicle, and at lower levels in GC. Blimp-1 has been reported to have roles for T cell function, and is present at high levels in antigen-experienced T cells ((Martins G 2008) review).

IgG1 switch transcript were mainly expressed in GC cells, but were not detectable at the GC-T zone interface ($p<0.001$ between GC-T and GC, Fig 4.27C). As the first exon of IgG1 ST is excised during switching, absence of expression may reflect successful Ig class switching. Accordingly, Iμ-Cγ1 rearranged Ig heavy chain transcripts appear in the GC-T zone interface. These indicate that cell successfully switched to IgG1 (Li SC 1994). J chain, essential for IgM and IgA polymerisation (Mestecky J 1971; Shaffer AL. 2004), was expressed highest in plasma cells. The GC-T zone interface upregulated J chain mRNA ($p<0.05$ between GC-T and GC). Therefore, GC-T zone area mainly includes plasmablasts, which have started to secrete switched antibody.

Germinal centre typical genes were expressed at low levels in the GC–T zone interface. Bcl-6 was almost undetetectable in the GC-T zone interface, whereas it was highly expressed in GC ($p<0.001$ between GC-T and GC). Bcl-6 was also detected in follicles, as described by Allman (1996), reporting that Bcl-6 mRNA is expressed in naïve B cells.

AID is highly expressed in GC cells, but expression is lost in GC-T zone interfaces ($p<0.001$ between GC-T and GC). Pax-5 is required for the early B-cell differentiation and maintenance of B-cell identity (Horcher M 2001; Nera KP 2006) but it is down-regulated in the terminal stages of B-cell differentiation to plasma cells (Souabni A 2002). As expected, Pax5 expression was lower in plasma cells, and high in follicles and GCs. In GC-T zone interfaces, Pax5 expression was lower ($p<0.001$ between GC-T and GC, Fig 4.27 C), reflecting plasmablast differentiation in this area. IRF4 expression in this area may repress Bcl-6 (Falini B 2000; Saito M 2007), Pax-5 shutting off further AID expression (Sciammas R 2006). Apart from Pax-5 downregulation, Blimp-1 induction (Shaffer AL 2002) is a possible mechanism for the termination of AID expression.

4.2.6.2 The expression of chemokines and chemokine receptors

Chemokines have an important role in guiding cell movement during the initiation of adaptive immune responses (Cyster 2005). CXCR5 is involved in the attraction of recirculating B cells and MZ cells B cells as well as a subset of primed CD4 T cells into B cell follicles (Schaerli P 2002; Ekland EH 2004; Cinamon G 2008). CXCR5 also helps direct cells to the light zone (Allen CD 2004). Not surprisingly, CXCR5 is expressed in follicles and also in the GC-T zone interface, with slightly lower levels in GCs and plasma cell areas (Fig 4.28 A). CXCL13 is highly expressed in follicles, and expressed in GCs and GC-T zone interfaces as well.

CXCR4 is expressed on GC B cells, especially on centroblasts (Caron G 2009) and plasma cells (Allen CD 2004). It is essential for dark and light zone segregation (Allen CD 2004). Further CXCR4 expression is implicated both in migration to the T zone (Casamayor-Pallejà M 2002) and from the T zone to the extrafollicular foci (Hargreaves DC 2001; Cyster 2003a). Upregulation of this gene was seen in the GC, and at lower levels in plasma cell areas and the GC-T zone interface (Fig 4.28 B). CXCR4 protein

expression in GC is low compared to the G–T zone interface (Fig. 4.18 H). The higher levels of CXCR4 mRNA in GC may be due to a low, but homogenous expression of CXCR4 in all cells in the GC.

CXCR4 is critical for normal homing of plasma cells to the red pulp in the spleen. Accordingly, the plasma cell area expressed a higher amount of the CXCR4 ligand, CXCL12 (SDF-1) (Fig 4.28 B). More importantly, high level of CXCL12 expression was detected at GC-T zone interfaces, although lower than that in the red pulp. This gradient may favour migration of new $CXCR4^{+}$ plasmablasts to the GC-T zone interface and then towards the CXCL12-rich red pulp.

CXCR7 (RDC1) is a scavenger receptor for CXCL12 (Balabanian K 2005; Thelen M 2008). In zebrafish it has been shown to produce a CXCL12 gradient needed to direct cell migration (Thelen M 2008). Detection of mRNA showed that CXCR7 was expressed mainly in follicles and less in GC cells ($p<0.05$ between GC-T and Follicle), compatible with CXCR7 producing a CXCL12 chemokine gradient over the GC with a CXCL12 sink towards the follicles (Fig 4.28 B).

Homing of T cells to T zone depends on CCR7 and ligands (CCL19 and CCL21) (Forster R 1996; Gunn MD 1999). CCR7 is further required for the localisation to and retention of B cells at the follicle-T zone interface following antigen-receptor engagement (Reif K 2002; Okada T 2005). As predicted, high amounts of CCR7 were seen in T zone, more interestingly it was also found in GC-T zone interface and plasma cell areas ($p<0.001$ between GC-T and GC, Fig 4.28 B). CCR7 expression has been shown to be important for follicular B cell migration (Ekland EH 2004; Okada T 2005), and low levels of CCR7 were detected in follicles.

CCL19 and CCL21, the two CCR7 ligands, are expressed in the T zone by radiation-resistant stromal cells. CCL21 is also made by HEV, whereas CCL19 can be produced by DC (Cyster 1999; Cyster 2005). High amounts of CCL19 and CCL21 expression were found in T zones and GC-T zone interfaces (Fig 4.28 B), leading to migration of cells to the GC-T zone interface.

CXCR3 is expressed on activated T cells, NK cells, and some antibody forming cells (AFC), and most T cells in inflammatory lesion express CXCR3 (Houser 2002). CXCR3 and the ligands CXCL9 and CXCL10 are expressed on early T zone B blasts (J. Marshall, personal communication), and also present on *in vivo* activated T cells (K. Serre, personal communication). To test whether CXCR3 has a role for the movement of IRF4+ plasmablasts to the GC-T zone border, CXCR3, and its ligands CXCL9, CXCL10, CXCL11 were assessed. CXCR3 expression was present in GC-T zone interfaces, T zones and plasma cell areas at similar levels, with slightly lower expression in the follicles (Fig 4.28 D). High CXCL9 expression is present in the plasma cell area and T zone, and moderate and lower levels were seen in GC-T zone interface and follicle respectively. Other ligands CXCL10 and CXCL11 (undetermined) showed similar abundances of expression throughout different areas. Whether any of the ligands of CXCR3 have a role in B blast or plasmablast migration remains to be studied.

4.2.6.3 The expression of cytokines and receptors

Most of the cytokines such as IL-6, IL-10, IL-4, IL-13, which can mediate the differentiation of GC B cells, were undetectable or present at very low amount in these microdissected areas at this time point. This could possibly be due to the low concentration of mRNA obtained from microdissected section or the choice of time point being suboptimal (data not shown here). IL-10Rα is expressed by a variety of murine cell types including B and T cells (Moore KW 2001), so interestingly, high amounts of IL-10Rα

expression were found on the whole spleen section, particularly in GC and GC-T zone interface areas. IL-10 mediates the development of B cells by signalling via IL-10Rα, and stimulates activation of other cell types (i.e. T cells) (Fig 4.29 A). This data helps clarify why small GC development and reduced plasma cell generation were detected in IL-10 KO mice.

IL-21 is mainly produced by GC T cells (Ettinger R 2008). Interestingly, a high level of IL-21 expression was detected in GC areas, and mediate amount at the GC-T zone interfaces in comparison to other areas ($p<0.001$ between GC-T and GC, Fig 4.29 A). Less $IRF4^+$ plasmablasts at the GC-T zone interface were found in IL-21/IL-21R deficient mice. These together could further indicate that the $IRF4^+$ plasmablasts seen at the GC-T zone interfaces mainly derive from GCs, particularly signals from T_{FH} cells in the response to the TD antigen. In summary, the selection process happened in the LZ for the final differentiation into the early plasmablasts.

4.2.6. 4 The expression of co-stimulatory signals

CD40L, primarily expressed on activated T cells, can cause activation of the resting B cells by directly binding to their CD40 receptor, and then guides the B cells through their differentiation programme, including rescue from apoptosis, differentiation into GC cells, isotype switching, selection, and maturation into memory cells (van Kooten C 2002). CD40L is expressed in the GC and T zone areas, as predicted. These is also low level expression at the GC-T zone interface (Fig 4.29 B), this is consistent with CD40L protein on T cells in the GC– T area interface in human tonsil (Casamayor-Palleja M 1995).

CD30 and OX40 have role for GC persistence and the generation of the early $IRF4^+$ plasmablasts (Fig 4.21), consequently it is not surprising that both CD30 and OX40 were detected at the GC-T zone border at lower levels than the expression seen in the T zone

(Fig 4.29 B).

4.2.6.5 The expression of survival signals for B cells

BAFF promotes B cell survival and Ig CSR (Mackay F 2002; Castigli E 2005). BAFF-R, one of three known receptors for BAFF, is associated with maintaining survival of recirculating and MZ B cells as well as GC B cells (Shulga-Morskaya S 2004; Rodig SJ 2005; Zhang X 2005). Clearly both BAFF and BAFF-R are highly expressed in GCs and follicles with intermediate expression in plasma cell areas. It is not surprising that GC-T zone interfaces also contain high amounts of BAFF and intermediate level of BAFF-R (Fig 4.29C.D). A proliferation-inducing ligand (APRIL) is critical for the plasmablasts in bone marrow (Belnoue E 2008). In comparison to the expression of BAFF and BAFF-R, APRIL has lower expression in spleen sections. The median level of APRIL in the red pulp area containing less $IRF4^{+}$ plasma cells, was higher than that in the follicle and plasma cell area. More interestingly, it is more strongly expressed at the GC-T zone interface than in the GC ($p<0.001$ between GC-T and GC, Fig 4.29 C).

While only BAFF binds BAFF-R, BAFF and APRIL exert effects through the cell-surface receptors including B-cell maturation antigen (BCMA) and transmembrane activator and calcium modulator and cyclophilin ligand interactor (TACI) (Xu S 2001; Seshasayee D 2003). TACI has been implicated in the early plasmablast differentiation (Zhang X 2005; Bossen C 2008) in TD and TI responses (von Bulow GU 2001). BCMA is reported to maintain plasma cells in the bone marrow (O'Connor BP 2006). While BCMA and TACI mRNA were absent from GCs, there was considerable expression of BCMA and TACI in plasma cell areas. Interestingly TACI is consistently expressed at the GC-T zone interface ($p<0.001$, Fig 4.29 D). This confirms published data on the role of these receptors in the plasmablast differentiation. Taken together, these data provide evidence that there may be a role for APRIL, and probably BAFF, via TACI and BAFF-R play a role in plasmablast differentiation at the GC–T zone interface.

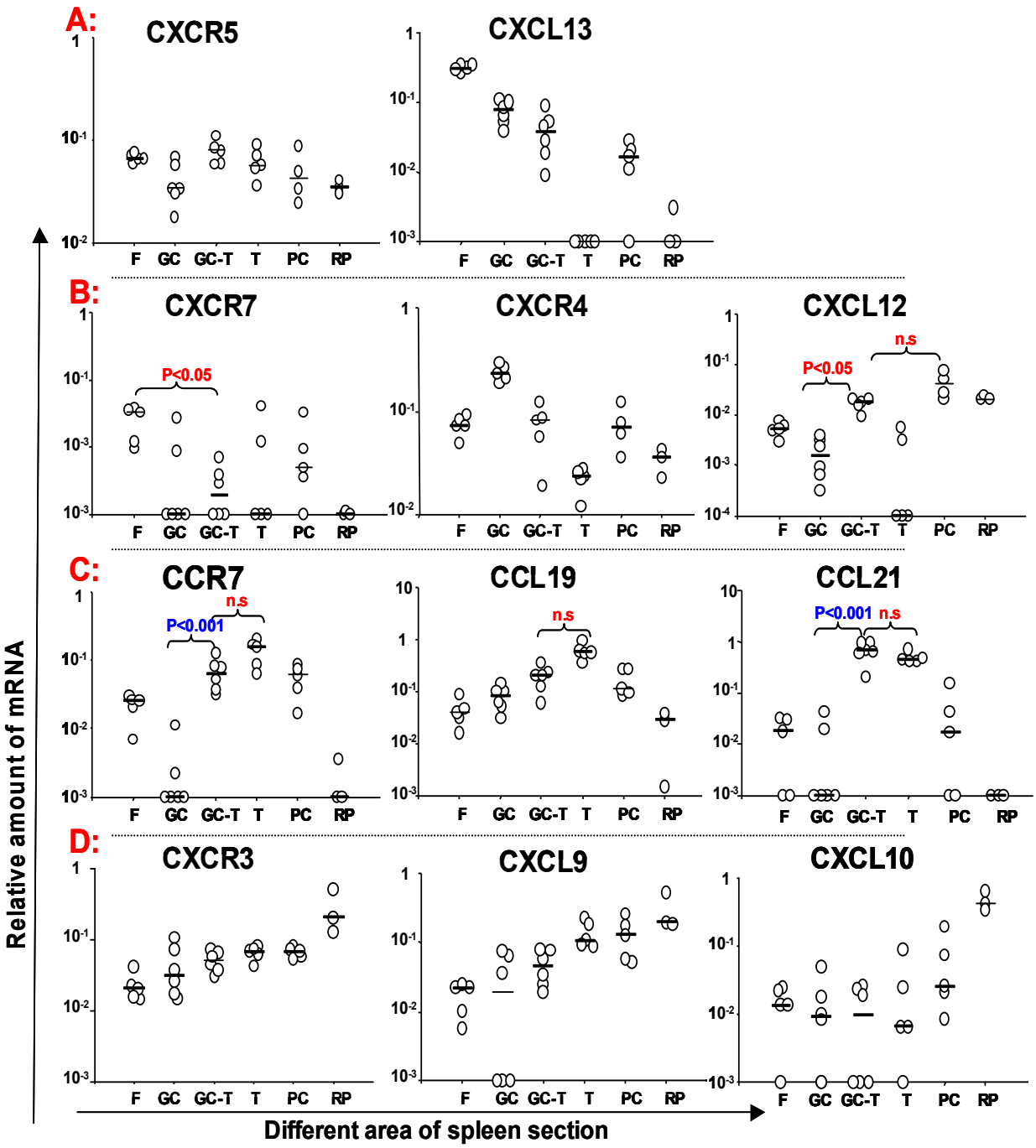

Figure 4.28: Gene expression of chemokines receptors and ligands at different areas of the spleen

The most important Chemokines associated with B cells homing and move out from GC to the red pulp were assess by RT-PCR. **A**: Chemokines guide lymphocytes homing and migration into follicles in the spleen. B: Chemokines guide B cells moving out from GC, and into the extrafolicular foci. **C**: Chemokines associated with T cells and dendritic cells homing in T zone. **D**: CXCR3 and its ligands CXCL9, CXCL10, CXCL11 (non-determined) were assessed as well due to they are involved in the plasmablats migration in the inflammation. Each spot represents one area of one spleen section. All values are relative to the house keep gene β_2-microglobulin. $p<0.05$/$p<0.001$ according to the Wilcoxon Mann-Whitney U test. This is representative of two individual experiment. GC-T: GC-T zone interface, GC: Germinal centre, T: T zone, PC: $IRF4^+$ plasma cell area, F: Follicle without GC. RP: red pulp with low/no $IRF4^+$ cells as control. n.s.: non-significance

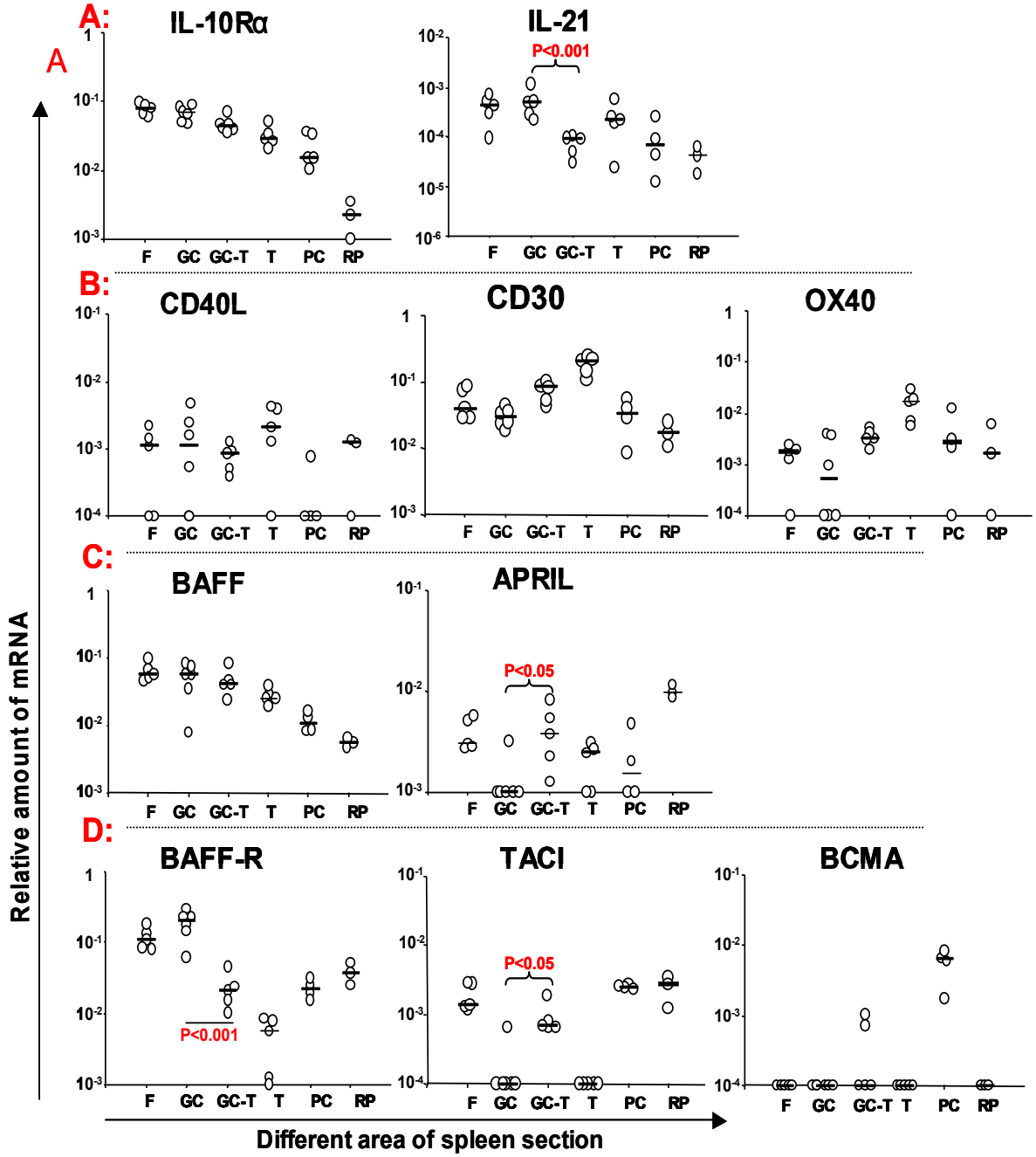

Figure 4.29: Assessment of more genes which may have roles for the production of IRF4$^+$ cells at the GC-T zone interface

Cytokines (**A**) and co-stimulatory signals (**B**) were assessed in the different compartments of the spleen section due to their effects for the production of IRF4$^+$ cells at the GC-T zone interface in the KO mice. **C.D**: typical survival signals and receptors for B cells were assessment. Each spot represents one area of one spleen section. All values are relative to the house keep gene β_2-microglobulin. $p<0.05/p<0.001$ according to the Wilcoxon Mann-Whitney U test. This is representative of two individual experiment. GC-T: GC-T zone interface, GC: Germinal centre, T; T zone, PC: IRF4$^+$ plasma cell area, F: Follicle without GC. RP: red pulp with low/no IRF4$^+$ cells as control. Most cytokines such as IL-4, IL-6, IL-10, IL-13 are undetermined.

4.3 Discussion

4.3.1 IRF4 is expressed shortly after B cell activation

The up-regulation of IRF4 is likely to be key in directing both CSR and commitment to differentiation to plasma cells, as absence of IRF4 is associated with the failure of both processes (Klein U 2006; Sciammas R 2006; Shaffer AL 2008). High level expression induces Blimp-1 and plasma cell differentiation, while intermediate levels are supposed to permit Ig class switching (Sciammas R 2006), a process that has been connected with GC B cell development (Cattoretti G 2006a). Surprisingly, IRF4 mRNA is significantly upregulated rapidly after activation in all B cells after immunization of QMxC57BL/6 mice with NP-Ficoll. mRNA expression peaks within 1h , and then quickly falls again. Further, IRF4 protein starts to be detectable on MZ B cells within 1h of immunization before they arrive in the outer T zone, however, the level of expression on B blasts is lower than that on plasmablasts. IRF4 is completely lost when B blasts differentiate into GC B cells, and highly upregulated in plasma cells. Similar data of low level expression of IRF4 in developing B blasts have been produced *in vitro* by the lab of Roger Sciammas (Keystone meeting B cells in context, Taos 2009). The early intermediate level IRF4 expression in the outer T zone is associated with *Aicda* mRNA expression in some B blasts 48 h after NP-Ficoll stimulation. Interestingly, these B blasts do not express *Bcl-6* mRNA. Therefore, the regulation of Ig class switching may be fundamentally different in these cells than in GC B cells (Marshall J, Zhang Y *et al.*, submitted for publication).

Differentiation towards two different cells types may be directed by bistable (switch-like) regulatory networks (Laslo P 2006). Both bistable and graded behaviour of IRF4 expression may control the onset and resolution of mixed lineage patterns during cell fate determination (Sciammas R 2006). IRF4 at low level expression may synergize with Pax-5 to induce AID (Gonda H 2003), while if expressed at higher levels it inhibits Bcl-6

(Falini B 2000; Saito M 2007) and induces Blimp-1 expression, suppressing Pax-5 and shutting off further AID expression (Sciammas R 2006). This expression of IRF4 may be due to NF-κB1 and STAT activation following BCR and CD40 signalling (Grumont RJ 2000; Saito M 2007; Lu 2008). While in response to NP-Ficoll, IRF4 is probably activated through continued BCR stimulation, which may induce other pathways than NF-κB1 (Bajpai UD 2000).

Furthermore, the immediate upregulation of IRF4 mRNA after stimulation suggests that IRF4 not only plays a key factor for post-GC plasma cell differentiation, but also has an important function for B cell activation. One direct target gene of IRF4 in activated B cells is MYC (Shaffer AL 2008), which is a key coordinator of cellular growth, metabolism and proliferation (Dang CV 2006). IRF4 and MYC mutually reinforce the expression of each other (Shaffer AL 2008; Shaffer AL 2009). A sharp increase in IRF4 binding to the MYC promoter was also detected after 3 and 20h of phorbol myristate acetate and ionomycin activation in human B cells (Shaffer AL 2008). GC cells need IRF4 for terminal differentiation to plasma cells (Cattoretti G 2006a). Function of IRF4 is associated with a change in interacting partners or with reduced interaction with transcriptional coactivator PU.1 (Mittrücker HW 1997; Tamura T 2005; Cattoretti G 2006a) in regulating B cell development and differentiation.

BCR interaction with NP-Ficoll may quickly activate upregulation of IRF4 in B cells reacting to NP-Ficoll in QMxC57BL6 mice. Similar data from genome-wide analysis of mouse B cells (*in vitro*) shows that genes have changed expression within 3 hours of BCR stimulation, particularly down-regulation of Pax-5, Bcl6, SpiB, and MITF, and activation of IRF4 (Hauser J 2009 in 2nd Eur. Cong. Immunol. Berlin). IRF4 contains several potential microRNA binding sites (Malumbres R 2009), and so miRNAs may have a role for the quick loss of IRF4. Microarray assays have revealed that several

miRNAs are differentially regulated upon stimulation with LPS or a combination of anti-IgM, anti-CD40 and IL-4 in the primary splenic B cells. IRF4 transcript was identified as a target whose expression is fine-turned by some miRNA that is induced upon antigen stimulation (Brandl 2009 7th B cell Forum Salzburg Austria). These studies together suggest that IRF4 immediately increased 1-2 hr after B cell activation, with subsequent reduction of expression maintained through the activation of miRNA.

miRNA-155, encoded within a region known as *bic* (*B* cell *i*ntegration *c*luster) (Lagos-Quintana 2002), also shows greatly increased expression in activated B cells, because it is induced upon B cell receptor (BCR) ligation (van den Berg A 2003) or antigenic stimulation (Rodriguez A 2007; Tili E 2007). A further study reported that BCR activation induced miRNA-155 expression though a conserved AP-1 element (Yin Q 2008). miRNA155 was found to attenuate NF-κB signalling in EBV infected cells (Lu 2008). The NF-κB signalling pathway may mediate IRF4 activation via BCR, CD40 and cytokine stimulation (Saito M 2007; Lu 2008). Loss of NF-κB signalling may also be responsible for the loss of IRF4 expression.

Different phases of IRF-4 induction may be activated by different signal transduction pathways. When NF-κB1 deficient B cells were transferred into normal hosts and challenged with NP-Ficoll, low level IRF4 expressions was undisturbed in the transferred B cells that arrived in the outer of T zone 1d after immunization. Plasma cell differentiation was strongly disturbed and so less plasmablasts were developing in bridging channels (L. George, unpublished). The results indicate that NF-κB1 activation is not the only way to induce IRF4 up-regulation in activated B cells in response to NP-Ficoll. Continued BCR stimulation may directly induce IRF4, other signalling pathways may exist. Nevertheless, NF-κB1 signalling may be essential for IRF4 high expression in the terminal differentiation of extrafollicular or post-GC plasmablasts

(Fig4.30).

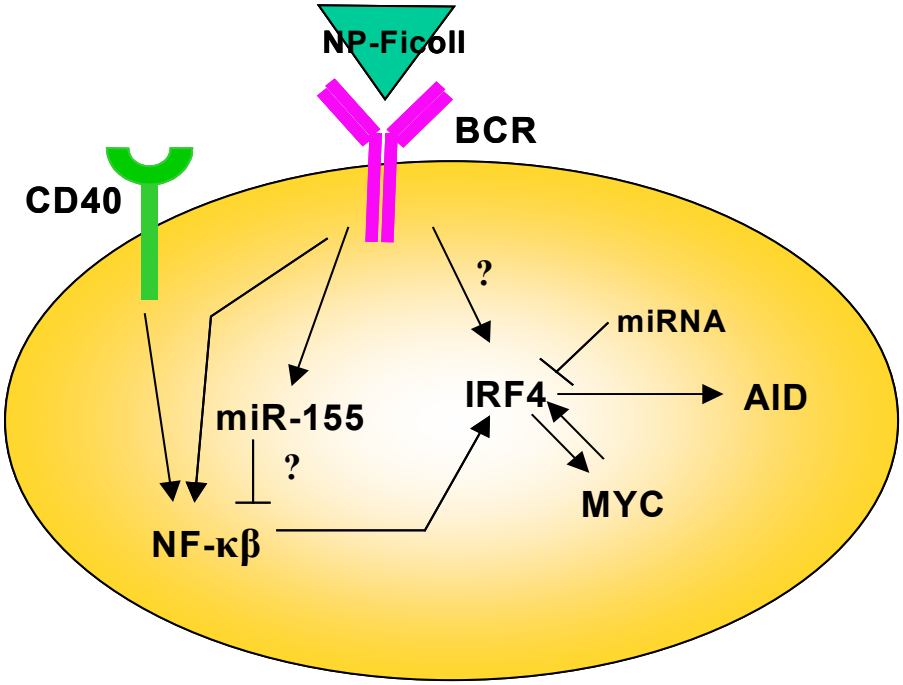

Figure 4.30: IRF4 regulatory network in activated B cells
IRF4 rapidly increases expression on B cells after NP-Ficoll stimulation by BCR, and then reduces and stays at low level may be due to some miRNA expression, leading to the induction of AID.

4.3.2 Plasmablast development in the GC response

The rapid increase in IRF4 mRNA and protein expression after stimulation suggests that IRF4 may be a good marker to detect B cell activation. Taking advantage of this, spleen sections were stained at different times after immunization, to identify the location of activated B cells in different areas of the splenic white pulp.

The experiments showed a distinctive distribution of IRF4 positive cells at different stages of the immune response (Fig. 4.15): Surprisingly IRF4 expression was already seen in some follicular naïve B cells and in T cells in the T zone. Occasional proliferation

in naïve T cells in the T zone has been described before (Luther SA 1997), possibly indicating homeostatic proliferation in naïve T cells. Similarly, naïve B cells have been shown to need occasional signals via their B cell receptor (Kraus M 2004). It is possible that IRF4 occasionally seen in follicular B cells corresponds to this process.

2-3 days after immunization, cells expressing IRF4 were mainly clustered at the outer edge of the T zone. Some, but not all, of these were T cells. In primary responses T cell priming is apparent around day 3 (Luther SA 2007), and IRF4 expression in T cells in the T zone is likely to represent this phase of T cell activation. Within several hours of encountering TD antigens, antigen-engaged B cells migrate to the B-T boundary area by rapidly upregulating CCR7 while keeping CXCR5 expression unchanged (Reif K 2002). Other chemokine signals and/or adhesion molecules may be also involved to guide and retain cells into the place for encounter with primed T cells (Okada T 2006). Primed T cells in the T zone can interact with B cells, exchanging cytokines such as IL-4 and induce B cell proliferation and Ig class switch recombination (Toellner KM 1998). In addition, in the outer T zone $CD11b^+$ DCs have been shown to be involved in B cell priming (MacPherson G 1999; Huang NN 2005). As discussed above, IRF4 is important for the regulation of B blast activation and Ig class switching (Huang NN 2005; Sciammas R 2006), Marshall, Zhang *et al*), and the intermediate level expression of IRF4 in B cells 3 days after immunization are likely to reflect IRF4 activating these processes.

After expression of IRF4 in B cells in the outer T zone, a wave of $IRF4^+$ cells was observed in follicles 3–4 days after immunization. Others recently showed that activated B cells move to the outer follicles, before the formation of GCs (Coffey F 2009; Gatto D 2009; Pereira JP 2009). The role of this differentiation phase is not clear.

Germinal centre B cells typically lose expression of IRF4 (Falini B 2000; Cattoretti G

2006a; Klein U 2008), and the transcription factors IRF8, Bcl6, and HoxC4 (Park SR 2009), cooperating with Oct2 are known to regulate the GC phenotype (Lee CH 2006; Lu 2008). $IRF4^+$ cells were seen at the GC-T zone interface at day4 post-immunization, just after GC formed. This population increased rapidly, reached peak levels at day5, and was lost after day5, even though GC still kept on developing for one more week. $IRF4^+$ B cells in the GC–T zone interface lost all GC markers investigated. The cells express high levels of IRF4, and this seems to induce the plasmablast differentiation program: The cells at the GC–T zone interface have acquired several plasma cell markers, such as Blimp-1 and CD138, showing that these cells are terminally differentiating plasmablasts (Turner CA Jr 1994; Shaffer AL 2002).

Recent experiments from our lab from lymph nodes 6-7 days after primary immunization with alum-precipitated NP-CGG could also identify an $IRF4^{high}$ plasmablast population in the GC–T zone interface. Microdissection and DNA sequencing could identify identical V186.2-DFL16.1-J_H2 sequences with identical CDR3 shared between GC B cells and plasmablasts from the adjacent GC–T zone interface (Yeo, MRe Thesis, UoB 2008). The clonal relation of these cells suggests that plasmablasts at the GC-T zone interface are descendants of B cells in adjacent GCs. This raises the question whether the GC-T zone interface is a pathway for B cells differentiating to plasmablasts exiting from immature GCs. The current model of GC differentiation proposes the exit of cells after T cell interaction from the light zone (MacLennan 1994). However, occasional exit of GC cells via the dark zone has been observed in intravital studies (Hauser AE 2007a).

Mature GCs from day 7 after immunization have few plasmablasts at the GC–T zone interface. Interestingly, $IRF4^+$ cells after day 8 clearly presented in the GC light zone. These cells expressed intermediated levels of IRF4 and were in close contact with FDCs and T_{FH} cells. IRF4 is absent from proliferating GC centroblasts and the majority of

centrocytes. Intermediate level IRF4 expression in the light zone may reflect the presence of several alternative, but not exclusive, differentiation processes occurring in the light zone B cells. The proximity of $IRF4^{+}$ cells to T cells suggests that they may have recently received T cell signals. IRF4 expression in the light zone may reflect plasmablast differentiation from selected light zone B cells (Angelin-Duclos C 2000; Klein U 2006; Cattoretti G 2006a). The differentiation to plasmablast cells from light zone selected GC B cells may reflect the appearance of a more stable long-term surviving GC output. However, few of these cells express high levels of IRF4 or Blimp-1. Therefore, the majority of IRF4 positive cells observed in light zones of mature GCs were either observed just before high level expression of IRF4 was reached, which is unlikely, or are involved in other differentiation processes. Alternatively these cells may have been selected to recirculate within the GC. Intravital studies using IRF4 reporter mice would be a way to test this. Finally, $IRF4^{int}$ cells may be precursors of memory B cells, as IRF-4 has been shown to be involved in the maturation of memory (Klein U 2006). The final wave of $IRF4^{int}$ B cells seen in follicles from day 8 after immunization may reflect the exit of these memory B cells from GCs. Memory B cells localization at the outer follicle have been detected in the recent research (Anderson SM 2007). Intravital studies have shown exit of GC B cells via this pathway (Schwickert TA 2007; Hauser AE 2007b).

Differentiation and activation of B cells seen in both LZ and GC-T zone interface indicate that there seem to be two pathways of successfully selected GC B cell exiting GC:

1) Early plasmablasts leaving via the dark zone-T zone interface rapidly producing GC derived plasma cells,
2) High affinity plasmablasts and memory cells being selected in the GC light zone and recruit to long-term responses that efficiently clear antigens.

How is the appearance of cells in these two microenvironments regulated? Differences in the selection threshold between immature and mature GCs may be responsible for the different migration patterns. Furthermore, availability of mature accessory cells may play a role.

As discussed in chapter 3, over the first days of the GC response the selection threshold changes dramatically. Appearance of plasmablasts at the basal part of the GC may be due to the low selection threshold in the early phase of the GC response. At this time antigens are already present on FDCs, but covered with very low affinity antibody. The high concentration of antigen in immune complex may be sufficient to induce T independent BCR stimulation in GCs. That the appearance of these cells may be independent from T cell signals can be concluded from the experiments, where T cell independent GCs were induced in QM mice after NP-Ficoll injection. On the other hand, significantly reduced $IRF4^+$ plasmablasts were seen at the GC-T zone interface after injection with high avidity immune complex, indicating that competition with antibody in immune complex on FDCs is important for the appearance of these cells. This would support the suggestion that rare static centrocyte-FDC contacts are sufficient to drive affinity maturation and antibody production (Figge MT 2008). Later in the response strong competition with high affinity antibody may mean that GC B cells receive little signal from antigens on FDCs. At this stage T cell help may become important, and therefore B cell activation events move to the light zone, where the maximum of T_{FH} cells are available (Gulbranson-Judge A 1996). These cells will provide signals that may induce different GC B cell differentiation outcomes (Butch AW 1993; Toellner KM 1995).

Different sites of B cell activation may be linked to different accessory cells. Whilst in the light zone signals will be provided by FDCs and T cells (MacLennan 1994; Liu YJ 1997a), the identity of cells providing signals for plasmablast differentiation in the GC–T

zone interface is not clear. In the red pulp plasmablasts differentiate into plasma cells, which are in close association with $CD11c^+$ DCs (de Vinuesa CG 1999; MacLennan IC 2003) and VCAM-1^+ stromal cells expressing CXCL12 and/or BAFF (Cassese G 2003; Cyster 2003a). These are critical for survival and homing of plasmablasts /plasma cells. The B-T zone boundary also contains many $CD11c^+$ DCs (Lindquist RL 2004), which were found to create tightly packed clusters with T and B lymphocytes in TD immune responses (Liu YJ 1991; Okada T 2006). They may be involved in the plasmablast differentiation similar their functions in red pulps.

Studies in human tonsil found that T cells in the GC–T area interface express preformed CD40L, similar to expression by T_{FH} cells (Casamayor-Palleja M 1995). CD40 ligation on B cells, *via* interaction with CD40L expressed on T_{FH} cells, has been shown to downregulate Bcl6 through NF-kB activation and consequent activation of IRF4 (Saito M 2007). The peak number of antigen specific T cells appears in the edge of T zone at the immature GC stage (Gulbranson-Judge A 1996). These $CD4^+$ T cells in the GC-T zone interface may provide similar functions as T_{FH} cells in light zones. This remains to be investigated by adoptive transfer and activation of labelled antigen specific T cells. Immunofluorescent staining further showed $IRF4^+$ cells were associated with BP-3^+ stromal cells and $CD3^-CD4^+$ Lti cells at the GC-T zone interface. Defects in the immune response to TI-2 antigens in $BP3^{-/-}$ mice suggested that BP-3 cells may contribute to splenic plasma cell niches even though the detailed mechanism was not clear (Itoh M 1998). $CD4^+$ Lti cells constitutively express a diverse set of TNF ligands (TRANCE, TNF-α, and lymphotoxins α and β, OX40L, and CD30L) (Kim MY 2003). Former work from the Lane group supports an important role for CD30L and OX40L from $CD3^-CD4^+$ Lti cells, enabling T cells to activate B cells during memory responses (Gaspal FM 2005; Lane PJ 2005; Lane PJ 2008; Withers DR 2009). We suggest that Lti cells may also mediate immune response of B cells by direct co-stimulatory signals to B cells (K. Perk,

Thesis UoB in preparation). *In vitro* research implied that TNF-α may enhance plasma cell survival (Cassese G 2003). In the B/T zone interface $CD3^{-}CD4^{+}$ LTi cells associate closely with $CD3^{+}CD4^{+}$ T cells, $CD11c^{+}$ DCs and VCAM-1 stroma cells which may be important in directing the migration of lymphocytes *via* chemokines (Kim MY 2003; Bekiaris V 2007; Kim MY 2007). Taken together, all these cells (DCs, BP3 cells, LTi cells) are possibly involved in the generation of the $IRF4^{+}$ plasmablasts at the GC-T zone interface by providing cytokines, co-stimulatory signals, and chemokines.

4.3.3 Effects of cytokines, co-stimulatory molecules and chemokines on the appearance of GC–T zone interface plasmablasts.

To study effects of factors inducing differentiation and migration of B cells into plasmablasts in the GC–T zone interface, two approaches were used. Firstly, animals with targeted deletions available in Birmingham or from collaborating partners were used. This approach allows the study of the deficiencies occurring in the absence of cofactors, but it can be difficult to conclude whether a cofactor is necessary during stages of the immune response, or much earlier in the development of the environment, in which the immune response occurs. Furthermore, it is not clear which cell type the defect is occurring in. For example, IL-6 deficiency has a large effect on appearance of GCs and delays onset of GC development, but it is not clear which cell is essential for production of IL-6, and whether IL-6 has effects directly on B cells, or on the differentiation of some accessory cell.

Therefore, to obtain information on the spatial relationships, and where cofactors are produced, laser microdissection followed by quantitative RT-PCR was used. This technique provides good information about the location of cytokines and chemokines at a certain time after immunization. Due to time constraints it was not possible to perform

time courses and get information about the temporal appearance of these factors, but this is a refinement to our experiments that may be pursued in the future. The problem with microdissection is that information of the location of a factor can be gained, but the spatial resolution is quite limited, probably 10-20µm. The subcellular resolution of immunohistology cannot be reached, and there is no connection between gene expression and individual cells. This is a problem if an area contains a mix of cells, as it is the case for the GC-T zone interface. A good example is CXCR4, which has been shown to be highly expressed in all GC–T zone interface plasmablasts. However, because the GC–T zone interface is a mix of plasmablasts and many T cells, the PCR signal from this area is weaker than that from GCs, where every cell expresses this receptor, but at lower levels. CD3ε and CD19 were used as controls to estimate the cellular composition in different microdissected areas. Still, microdissection has advantages over immunohistology, as soluble factors i.e. chemokines and RNAs, for which no antibodies are available, such as switch transcripts can be detected.

4.3.3.1 Cytokines and costimulatory molecules

IL-4 and IL-13 are likely to have effects on the interaction of GC T cells and B cells, and are know to have effects on Ig class switching (Cunningham AF 2002). However, both cytokines are able to replace each other in single gene deficient animals (Cunningham AF 2004a), and therefore effects on plasmablast differentiation in single gene deficient animals were not seen. Using IL-4Rα deficient animals overcome this problem, here GC onset is delayed, and plasmablast output from GCs 5 d after immunization is negligible. mRNAs for IL-4 and IL-13 are produced at very low levels in Th2 differentiated cells (Serre K 2009), and therefore they are not detectable by microdissection.

As mentioned above IL-6 has various effects on B cell differentiation. GC differentiation was delayed, and GC plasmablast output was strongly suppressed. IL-6 has been reported

to directly stimulate activated B cells to secret antibody (Randall TD 1993). In addition, a recently study from Wu *et al* found that IL-6 derived from immune complex-activated FDC could promote the GC reaction, somatic hypermutaion and IgG production(Wu Y 2009). *In vitro*, IL-6 can stimulate IL-6 deficient GC cells isolated from KO mice into cell cycle progression, and increase antibody production (Kopf M 1998). IL-6 mainly acted on GC B cell directly via IL-6R and gp130, and indirectly by promoting the C3 production in the local environment (Kopf 1998). In addition, IL-6 has also a profound effect on survival and proliferation of CD4 T cells and Th17 differentiation (Dienz O 2008), and in this way may reduce T cell help for B cells, further declining the GC reaction and plasma cell production.

IL-10 has a broad effect in regulating the T cell-mediated immune response (Moore KW 2001; Maynard CL 2008). As for IL-6, the GC response was reduced, and output of $IRF4^+$ plasmblasts at the GC-T zone interface significantly reduced. For some time IL-10 has been known as a factor that can induce plasma cell differentiation. IL-10 has been reported to enhance proliferation and differentiation of B cells (Moore KW 1993). It was originally known as plasma cell differentiation factor (Maliszewski CR 1993; Arpin C 1995; Choe J 1998). IL-10 is produced by GC T cells (Butch AW 1993; Toellner KM 1995), and can lead to differentiation of GC B blasts to plasma cells. The defect in IL-10 KO mice possibly suggests a role of GC T cell–centrocyte interactions for the differentiation of GC-derived plasmablasts.

Similar to IL-10, IL-21 is produced by T_{FH} cells. IL-21 has a minimal effect for T_{FH} expansion, and development, but is important to regulate growth and differentiation of T and B cells directly (Ettinger R 2008; Zotos D 2010). Particularly IL-21 is required for the efficient formation of plasma cells (Ozaki K 2002; Odegard JM 2008). IL21 from GC T cells controls the expression levels of Blimp-1 and Bcl-6 via STAT3 (Ozaki K 2004;

Calame 2008; Haynes 2008; Kwon H 2009). IL-21/IL-21R KO mice have fewer NP^+ plasmablasts/plasma cells in red pulps after NP-KLH injection (Zotos D 2010). The result that far fewer GC-derived $IRF4^+$ plasmablasts develop at the GC-T zone interface supports the idea that T_{FH} cell - GC B cell interaction has a role in the differentiation of the $IRF4^+$ plasmablats at the GC-T zone interface.

The IL-10 family member IL-22 has been mainly described as having roles in the early host defense against microbial pathogen, tissue repair, wound healing and the homeostasis of epithelia (Wolk K 2006; Zenewicz L 2007; Zheng Y 2007; Zheng Y 2008; Pickert G 2009). IL-22 receptor expression has been described so far on epithelial cells, but not on B cells (Wolk K 2004; Jones BC 2008). IL-22 deficiency resulted in increased numbers of GC associated plasmablasts. IL-22 mRNA was amplified from GCs and in the GC–T zone interface. IL-22 is not only produced by T helper cells (Kreymborg K 2007; Colonna 2009), but also is produced by adult lymphoid tissue inducer (Lti) cells, which are located in the GC-T zone interface (Takatori H 2009). As adult Lti cells express further signals (e.g.CD30L, OX40L) that influence B cell differentiation (K. Perks, Thesis UoB), both GC T cells and Lti are candidates as producers of this cytokine. IL-22 deficiency also resulted in increased numbers of T cells in LZ. Therefore, the increased production of plasmablasts may be also due to an indirect effect, as increased numbers of T cells may increase availability of positive selection signals.

BAFF and APRIL and their receptors play important roles in the differentiation of B cells and plasmablast (Moisini I 2009). BAFF is also important for the formation of follicular dendritic cells, and can influence Ig hypermutation (Kalled 2006). APRIL has been shown to be associated with plasma cell differentiation and loss of proliferation (Mohr E 2009). Interestingly, TACI was strongly upregulated at the GC–T zone interface. TACI interacts with APRIL, which is produced by Lti cells (Kim MY 2008). And APRIL was detected at

the mRNA level in the GC–T zone interface. Plasmablasts leaving from this area and then migrating to the red pulp express further TACI and seemingly upregulate BCMA, which may interact with APRIL, produced in the same area. BAFF and BAFF-R seem to be more important in the GC, and lost after cells leave the GC.

Adult $CD4^{+}CD3^{-}$ LTi cells also express CD30L and OX40L, mediating T cell memory. Signals through CD30 and OX40 have been shown to be important for long term development of GCs, and B cell memory (Gaspal FM 2005; Lane PJ 2006). CD30 is expressed on activated murine T and B cells, and stromal cells in murine lymphoid tissues (Seko Y 1999; Bekiaris V 2007). CD30 has complex functions, depending on the cell type, stage of development, and presence of other stimuli (Review on Kennedy 2006). For B cells, CD30L expressing cells in the presence of cytokines (IL-2, IL-4 and/or IL-5) induce B cells to proliferate, differentiate and produce antibodies *in vitro* (Shanebeck KD 1995). CD30 co-engagement may downreguate isotype switching in human B cells (Jumper MD 1995; Cerutti A 1998). OX40, initially identified as T-cell activation marker, can promote effector T-cell expansion and survival, and enhance cytokine production, and but not control initial activation and proliferation (Watts 2005; Croft M 2009). OX40/OX40L can augment the accumulation of T cells in B cell follicles (Brocker T 1999; Fillatreau S 2003). OX40 can have roles in the maintenance of follicular T cells (Gaspal FM 2005). A study in our lab on effects on CD30 and OX40 deficiency on responses to TI-II antigens (e.g. NP-Ficoll) showed that B cell expressed CD30 and OX40, possibly interacting with CD30L and OX40L on LTi cells, have opposite effects on plasmablast differentiation: whilst CD30 deficiency resulted in faster onset of plasmablast responses, OX40 deficiency resulted in lower and delayed responses (K. Perks, thesis UoB, in preparation). Our experiments showed similar effects of CD30 and OX40 deficiencies on the appearance from plasmablasts from GCs, which would be compatible with a role of Lti cells located in the GC–T zone interface for differentiation

of GC-derived plasmablasts.

Germinal centre was found to form in OX40 KO mice, but significantly bigger than WT after SRBC stimulation. B cells are activated at the same speed as T cells are primed (Toellner KM 1996), and so activated B cells could establish a GC reaction with follicular T cells at the beginning. OX40 deficiency impaired the late proliferation of effective $CD4^+$ T cells and shortened T cell survival, and so GC B cells just did abnormal expansion without effective follicular T cell help, resulting in less GC B cells than could normally developed, differentiation into plasmablasts. As prediction, much less $IRF4^+$ cells were detected at the GC-T zone interface in OX40 deficient mice. Less output further helps explain the larger GC size. Possibly, the data will support the suggestion that $IRF4^+$ plasmablasts mainly derive from GC B cells, which need contact with T_{FH} cells for proliferation and selection in response for TD antigens.

More severe impairment of the B/T segregation and much smaller T zones were detected in CD30OX40 dKO mice. Abnormal and bigger GC formed, and much less $IRF4^+$ plasmablats at GC-T zone interface, and $IRF4^+$ plasma cells in the red pulp, were detected. Both OX40 and CD30 were not required for the GC initiation and plasma cell production. OX40 and CD30 could have overlapping functions in the maintaining of CD4 T cells via OX40L and CD30L expressed on LTi cells in the light zone and the B/T interface (Lane PJ 2005). CD30 signals are essential for podoplainin expression on T zone $VCAM\text{-}1^+$ stroma cells in maintaining the B/T segregation (Bekiaris V 2007), and so CD30 deficiency impaired the functions of $VCAM^+$ stroma for T cell homing. $CD11c^+$ DC expressing OX4OL, could lead CD4 T cells to accumulate in the follicle (Brocker T 1999). Therefore CD30OX40 dKO could directly affect the normal development of CD4 T cell, and reduce the functions of LTi cells, DCs, and stromal cells for T cells via ligation with OX40L and CD30L. These factors together aggravate the impairment of B

cell responses.

In conclusion, cytokines IL-10 and IL21, possibly derived from GC T cells, seem to have a role for the differentiation of GC-derived plasmablasts. Lti cells in the GC–T zone interface, are stronger candidates for a role for providing signals directly in the GC–T zone interface. They are known to produce APRIL, IL-22, OX40L, CD30L (Kim MY 2008) (summary on Fig 4.31). An argument for T cells not being directly involved in the differentiation of these cells is that AID mRNA and IgG1 switch transcript production was very low. Therefore the GC associated plasmablasts do not seem to be under the influence of T cell derived Ig class switch inducing cytokines, such as IL-4 and IL-13, although a high proportion is Ig class switched, as seen by the high levels of Iμ-Cγ1 recombined heavy chain transcripts.

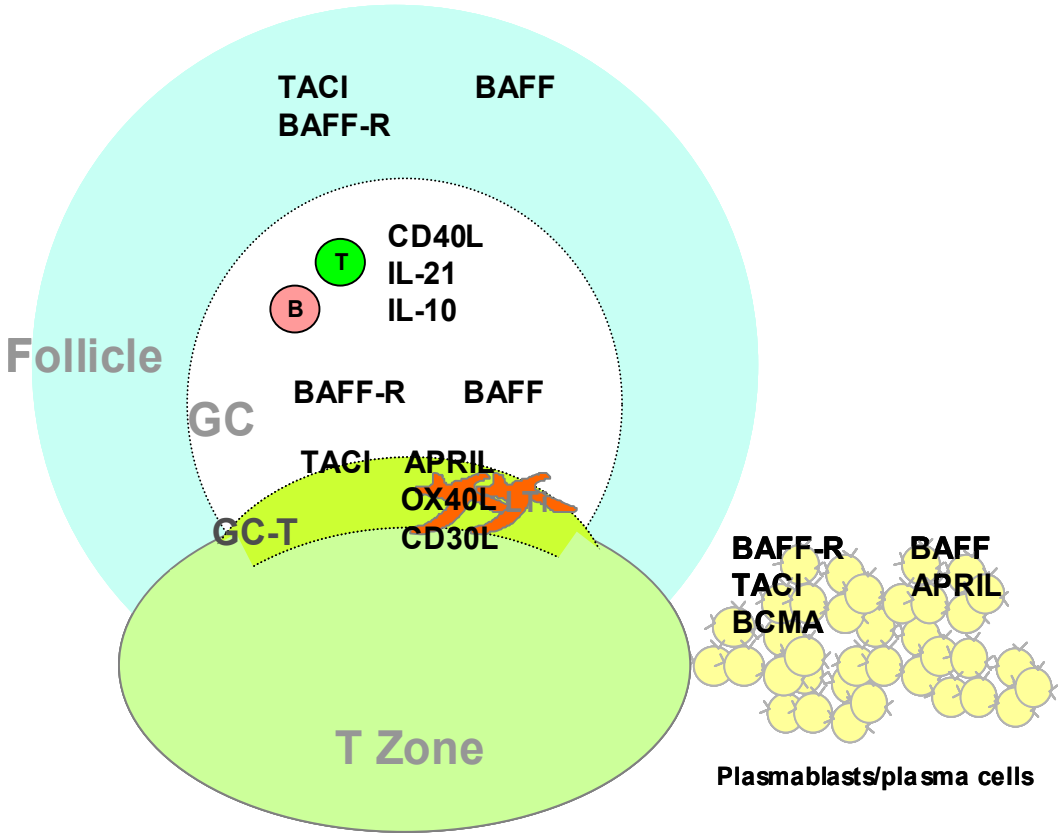

Figure 4.31: The expression of cytokines, co-stimulatory signals for B cell differentiation and survival at the different areas of the murine spleen at the stage1 of GC development

GC B cells get helps from TFH cells by co-stimulatory signal such as CD40L, and cytokines i.e. IL-21 and IL-10, for the survival and differentiation. BAFF high expression in GCs promote B cells survival and Ig CSR, and provide the survival signal plasma cells in the red pulp. Particularly, LTi cells produce OX40L and CD30L in the GC-T zone interface, which are important for the proliferation of GC B cells and differentiation into plasmablasts. APRIL expressed by LTi cells may provide the similar functions for IRF4^{+} plasmablasts at the GC-T zone interface as in the red pulp. TACI also expresses at the detected level at this area.

4.3.3.2 Chemokines

Chemokines and their receptors play important roles for the cell activation and migration. Within secondary lymph organs the majority of B cells and a fraction of primed CD4 T cells localize to follicles dependent on their expression of CXCR5 and the localized expression of its ligand CXCL13 by follicular stromal cells (Müller G 2003; Cyster 2005). Interestingly, CXCR5 and CXCL13 mRNA were found in high concentrations in the GC–T zone interface, indicating that plasmablasts exiting from the GC is not mediated by loss of sensitivity to this chemokine. IRF4 can promote expression of CXCR4 (Johnson K 2008). CXCR4 is a significant chemotactic factor for plasma cell precursors to home in the red pulp and subsequently in bone marrow. Its ligand CXCL12 is strongly expressed by VCAM-1^+ stromal cells, and also was reported to enhance plasma cell survival *in vitro* (Cassese G 2003). The scavenger receptor binding CXCL11 and CXCL12 (Balabanian K 2005; Burns JM 2006; Thelen M 2008) can remove soluble CXCL12 without signalling, this way producing a CXCL12 gradient (Naumann U 2010). Accordingly, CXCR7 was found in all microdissected follicular areas, and CXCL12 was found to be strongly expressed in the GC–T zone interface, with little expression in the GC. A CXCR7 induced sink in the follicle may produce a strong CXCL12 gradient, which would induce a quick transfer of CXCR4 high plasmablasts towards the T zone. Increased CCR7 expression on cells (such as naive T cells, some B cells, DCs) favours them to localize in T zone, and CCL19 and CCL21 are the only ligands for CCR7, expressed in T zone (Cyster 2005). CCR7 and CCL21 are not produced in the GC environment, but strongly expressed in the GC–T zone interface. A summary of the chemokine data is given in Fig. 4.32

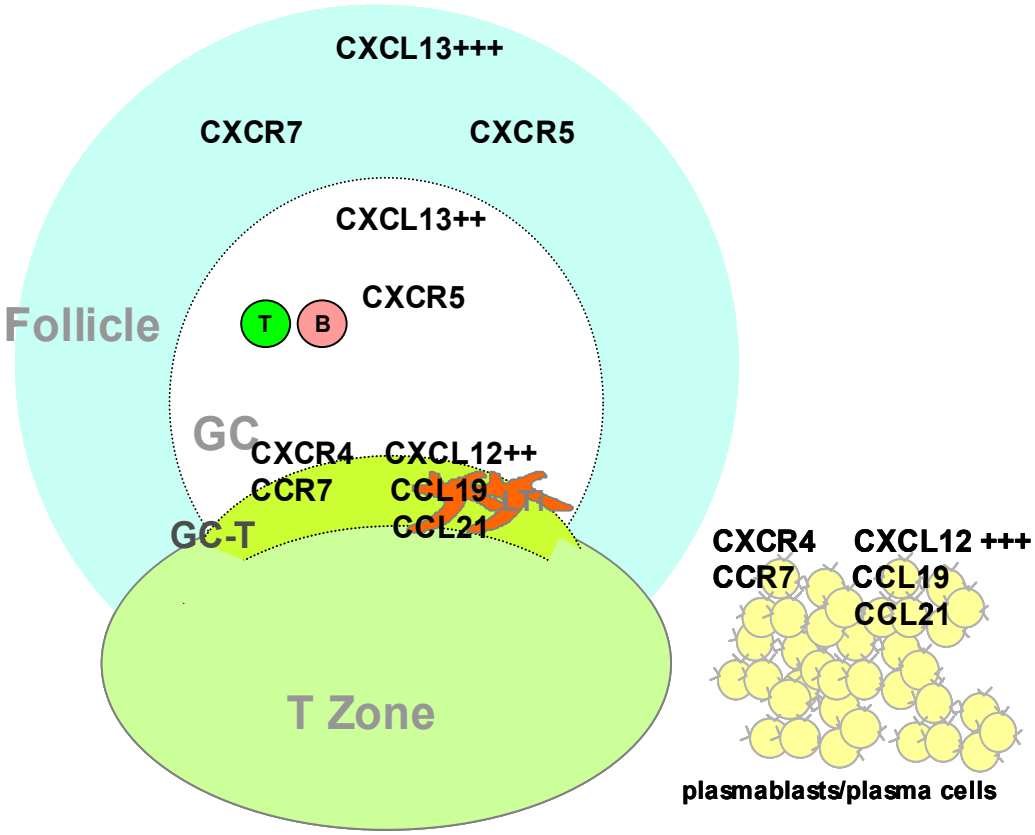

Figure 4.32: The expression of main chemokines and their receptors at the different areas of the murine spleen at the stage1 of GC development

The expression of CXCL13 and CXCR5 in follicles and GCs lead B cells to localize in GCs. The upregulation of CXCR4 on the IRF4+ plasamblasts guild cells move into the CXCL12 rich area. CCR7 expression also helps the homing of IRF4+ plasmablasts at the GC-T zone interface. GC-T: GC-T zone interface. GC: Germinal centre. LTi: Adult lymphoid inducer cells

Conclusion

T-cell dependent responses are initiated when naïve B cells encounter antigen and migrate to the outer T zone (Cyster JG 2002; Campbell DJ 2003), where they become fully activated by interacting with antigen-specific $CD4^+$ T helper cells and antigen-presenting cells.

Activated B cells may adopt one of two fates: either migration into the extrafollicular foci to differentiate into short-lived plasma cells, or movement into the follicles to form germinal centres in the primary follicle (Jacob J 1991; Liu YJ 1991). After initial phase in which B blasts expand in-between FDC in follicles, mature germinal centres develop which consist of two histologically distinct zones: the dark zone and the light zone (Stage 2) (MacLennan IC 1990). In the dark zone, centroblasts proliferate and diversify their Ig variable genes by the process of somatic hypermutation (SHM). B cells then test their modified antibodies for improved antigen binding in the light zone. The data presented here are compatible with centrocytes having to compete for antigens with antibody in immune complexes on FDCs to receive survival signals. There may be additional competition between B blasts with processed antigen for help from T_{FH} cells (Meyer-Hermann ME 2006; Allen CD 2007b). B cells that are unsuccessful die by apoptosis (Liu YJ 1997a) and are taken up by macrophages. Centrocytes that are selected are thought to migrate back to the dark zone where they undergo further proliferation and SHM (MacLennan 1994). Alternatively, selected centrocytes differentiate into plasma cells or memory cells (MacLennan 1994).

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Germinal centres have been described to develop in stages: Whilst stage 1 germinal centres developing in the first week after immunization mainly contain germinal centre founding blasts, which proliferate in FDC networks in secondary follicles, stage 2

germinal centres have separated into dark zones with highly proliferative centroblasts and light zones containing centrocytes, FDCs and apoptotic bodies (MacLennan IC 1990) (Fig. 5.1).

Initially the B blasts in early germinal centres will only be competing with low affinity antibody, which is either natural antibody cross-reacting with the antigen, or antibody produced from non-affinity-matured plasma cells from the early extrafollicular response (Tew JG 1997). Therefore, many B blasts will easily receive positive selection signals even after short contact with antigen on FDCs (Allen 2007a, Schwickert TA 2007, Figge 2008), and may be less dependent on the help from T_{FH} cells in the light zone. The migration of primed T helper cells from the T zone to follicles is only complete 3 days after the onset of germinal centre development (Gulbranson-Judge A 1996). More T cell help may be available at the GC–T zone interface (Fig. 5.1 A). Other accessory cells providing signals for the development, migration and survival of the early GC derived plasmablasts may be adult LTi cells, located in close contact with stromal cells in the GC-T zone interface. Selected cells may rapidly differentiate into early plasmablasts upregulating IRF4 at the GC-T zone interface. From there they will leave along the outer T zone, and finally into the red pulp (Fig. 5.1A). Selected cells may also do recycling and affinity maturation, which will lead to an increasing affinity of the plasma cell population outside germinal centres. This high affinity antibody will produce a positive feedback by replacing the low affinity antibody in the GC (Tew JG 1997), resulting in raising the the selection threshold for centrocytes (Fig. 5.1 B). As antigen-derived signals from FDC at later stages will be more difficult to obtain, T cell help may become more important (Meyer-Hermann ME 2006; Allen CD 2007b). More T_{FH} cells appear in GCs at this stage (Gulbranson-Judge A 1996; Ansel KM 1999). This will reduce plasmablast output from the GC (Fig. 5.2). Because B cells need more time to acquire appropriate selection signals from FDCs and T_{FH} cells, a wave of light zone emigrant develops. Light zones are

characterized by centrocytes that less proliferate, apoptotic cells and T_{FH} cells (MacLennan IC 1990). Recent intravital studies have shown that germinal centres develop in stages. Initial two-photo imaging experiments showed that GC B cells travel between FDC, and GC B cells transit between the dark and light zone evenly in both directions (Schwickert TA 2007; Allen CD 2007a; Hauser AE 2007a). Recent studies at later stages after immunization show that B cells migrate preferentially from the dark zone to the light zone, and fewer cells move back (Schwickert TA 2009), showing that in mature germinal centres the light zone represents a sink with a net loss of cells, indicating a higher selection pressure at later stages of the response. Latest intravital studies have shown that the speed of GC B cell migration changes considerably over the course of a GC response: whilst GC B cells in immature GC move slowly, mature GC B cells show rapid movement between FDCs and T cells (A. Hauser, DRFZ, Berlin, personal communication). This may be explained by changes in the availability of antigen and the need for additional signals from other cell types (i.e. FDC and T cells). The rise in antibody affinity will ultimately lead to a non-sustainable selection threshold, which may end the germinal centre reaction or lead to stage 3 germinal centres, which consist of small foci of proliferating cells (MacLennan IC 1990). These may be sustained through repeated restimulation of recirculating memory B cells on high affinity immune complexes deposited on FDC.

A positive feedback loop, where the germinal centre selection threshold is controlled by antibody from germinal centre derived plasma cells will stabilize and prolong the germinal centre reaction, as the slow increase in selection threshold will lead to efficient centrocyte selection over a longer period. This may result in a more efficient affinity increase at the beginning of the response, but also lead to higher final affinities. Furthermore, antibody feedback may lead to competition between unrelated germinal centres. Ultimately, this mechanism may abort the germinal centre reaction at the time

when hypermutation and Darwinian selection have led to optimal affinity, and no further affinity improvement can be gained by further mutation.

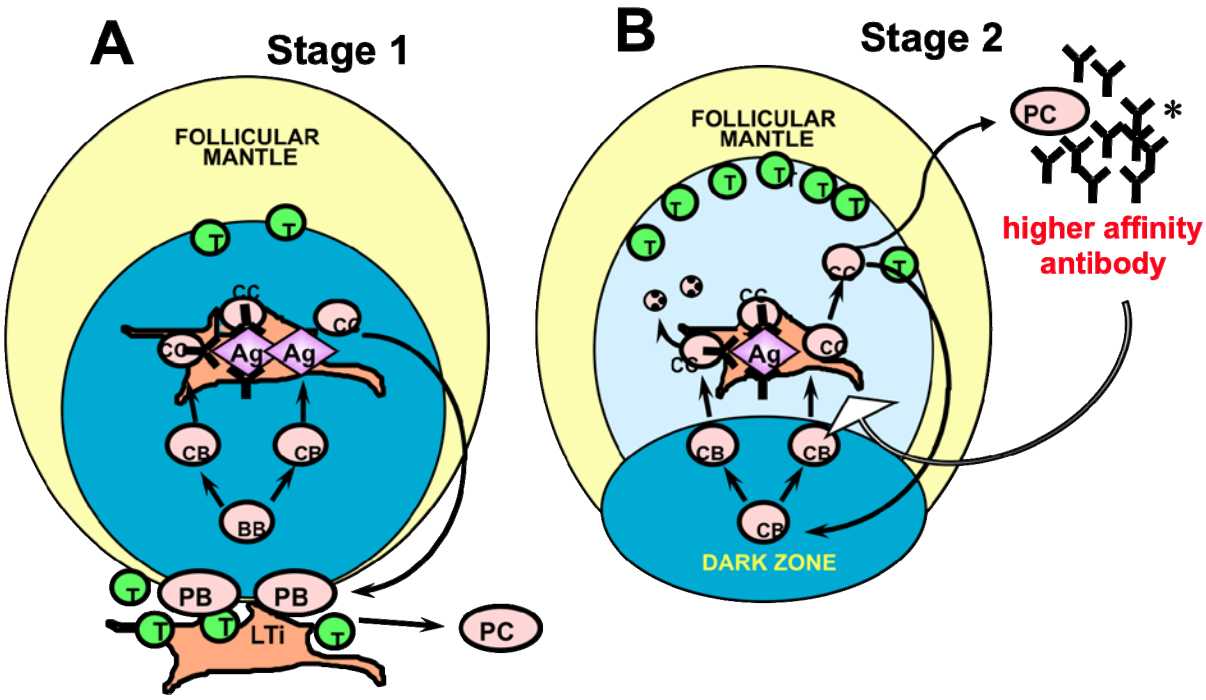

Figure 5.1: Development of germinal centres (adapted from MacLennan, I.C. et al (1990). The evolution of B-cell clones)
A: Immature germinal centres consist of proliferating B blasts the fill the FDC network. At this stage antigen is deposited in the form of immune complex with low affinity antibody. B blasts at this stage may have easy antigen access, and FDC – B blast interaction may be sufficient to produce positive selection. Accessory signals may be provided by adult LTi in the GC – T zone interface and by primed antigen-specific T cells, which may be available in high numbers in the outer T zone. **B**: Mature germinal centres suffer from a high selection threshold controlled by antibody produced by germinal centre derived plasma cells. This will lead to the stringent selection on FDC. As signals from FDC will be less easy to obtain, T cell help may become more important, which may induce different selection outcomes, such as differentiation of memory B cells, high affinity long-lived plasma cells or further recirculation. BB: B Blast; CC: Centorcyte. Ag: antigen; FDC: Follicular dendritic cell; PB: Plasmablast, LTi: Adult Lymphoid tissue inducer cell; T: $CD4^+$ T helper cell, CB: Centroblast, PC: Plasma cell.

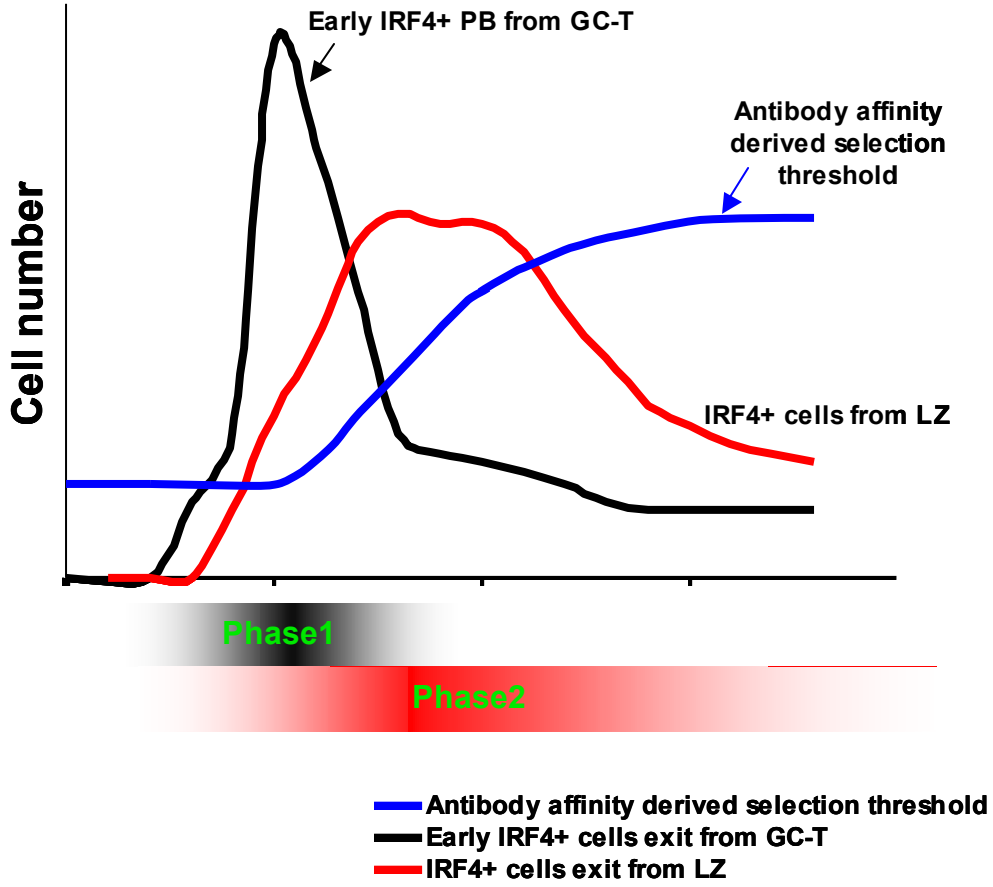

Figure 5.2: The antibody affinity in ICs in the GC and GC emigrants during the GC reaction

At the beginning of GC development, low affinity antibody in immune complexes (ICs) on FDCs is natural antibody produced from the early extrafollicular response (blue line). B blasts in germinal centres easily gain access to antigen, and are successfully selected to recycle or differentiate into the early $IRF4^{+}$ plasmablasts that exit from the GC-T zone interface (black line). As the antibody affinity from GC output increases, higher affinity antibody will positively feedback on the GC selection threshold (blue line). This will lead to reduce plasmablast output from the GC, and induce stringent selection of B cells, involving interaction with T_{FH} cells in the germinal centre light zone (red line).

Appendix

Table1: Primers for genotyping QMxB6

Primer		Sequence
β-actin	Fwd	GTGGGGCGCTCTAGGCACCA
	Rev	CGGTTGGCCTTAGGGTTCAGGGGGG
NP	Fwd	TTCAGAGGTTCAGCTGCAGCAGT
	Rev	CTYACCTGAGGAGACDGTGA
Jh	Fwd	TCGCCTTCTATCGCCTTCTT
	Rev	GCCCCAACTATCCCTCCA
JhWT	Fwd	AGCTCCCATACTTCATGGCCA
	Rev	TTGGTCCTGAAGGCCCAGAAT
Kappa	Fwd	CTGGTGCTTTACGGTATCGC
	Rev	GTAGAAGGCTCAAGGTTGTG
Kappa WT	Fwd	TGCTGCACCAACTGTATCCA
	Rev	CAGGACGCCATTTTGTCGT

Table2: primers for genetyping Cγ1-Cre$^{+/-}$× R26 eYFP$^{+/-}$

Primers for Cγ1-Cre$^{+/-}$
Primer A: IgG1 Kpn1Fw: TGTTGGGACAAACGAGCAATC
Primer B: Cg1-cre Fw: GGTGGCTGGACCAATGTAAATA
Primer C: IgG1 Rev3: GTCATGGCAATGCCAAGGTCGCTAG
Cg1-Cre allele using primers: Cg1-cre fw+ IgG1-Rev3: 455 bps
Cg1 wild-type allele using primers IgG1 Kpn1 fw+ IgG1-Rev3: 240 bps

Primers for R26eYFP$^{+/-}$
Primer1: oIMR0883: AAAGTCGCTCTGAGTTGTTAT
Primer2: oIMR0316: GGGGCGGGAGAAATGGATATG
Primer3: oIMR4982: AAGACCGCGAAGAGTTTG TC
Wildtype using primer1+Primer2: 600bps
Mutant using Primer1+ Primer3: 320bps

Table3: Sequence of primers and probes used for RT-PCR

Primers	Forward primer sequence	Reverse Primer Sequence	Probe sequence
IRF4	GGAGGACGCTGCCCTCTT	TCTGGCTTGTCGATCCCTTCT	FAM AGGCTTGGGCATTGTTTAAAGGCAAGTTC BHQ1
Blimp-1	CAAGAATGCCAACAGGAAGTATTTT	CCATCAATGAAGTGGTGGAACTC	FAM TCTCTGGAATAGATCCGCCA MGB
XBP-1	GGTGCAGGCCCAGTTGTC	CAAAAGGATATCAGACTCAGAATCTGAA	FAM TCCCATGGACTCTGACACTGTTGCCTC MethRed
AID	GTCCGGCTAACCAGACAACTTC	GCTTTCAAAATCCCAACATACGA	TET GCTTTCAAAATCCCAACATACGA BHQ1
Bcl-6	CAGACGCACAGTGACAAACCA	ACTGCGCTCCACAAATGTTACA	CalFlour 560 ACTGCGCTCCACAAATGTTACA BHQ1
PAX 5	GCTGTTGGCAGAGCGAGTCT	AGCTGGGACCGGCTGATT	FAM ACACTGTGCCCAGCGTCAGCTCC MethRed
IgG1 ST	CGAGAAGCCTGAGGAATGTGT	GGAGTTAGTTTGGGCAGCAGAT	FAM TGGTTCTCTCAACCTGTAGTCCATGCCA BHQ1
IuCu	TCTGGACCTCTCCGAAACCA	CCAGGGTCACCATGGAGTTAGT	FAM TGCCAAAACGACACCCCCATCTGT MethRed
J Chain	GTCCTGGCCATTTTTGTTAAGG	ACATGCATTTGTTGTCAGCAAGA	FAM TGGTCGCTTCGTCGTCACCTGTTACA MethRed
CXCR5	GCTCTGCACAAGATCAATTTCTACTG	CCGTGCAGGTGATGTGGAT	FAM CCATCGTCCATGCTGTTCACGCC BHQ1
CXCL12	CAAGCATCTGAAAATCCTCAACAC	CACTTTAATTTCGGGTCAATGCA	FAM TGCACGGCTGAAGAACAACAACAGACAA BHQ1
IL-4	GATCATCGGCATTTTGAACGA	AGGACGTTTGGCACATCCAT	FAM TGCATGGCGTCCCTTCTCCTGTG MethRed
IL-21	ACACCCAAAGAATTCCTAGAAAGACTAA	TGCATTCGTGAGCGTCTATAGTG	FAM AGCATCTCTCCTAGAACACATAGGACCCGAAGAT BHQ1
CD40L	CTCAAATTGCAGCACACGTTGTA	TCAAGCATTACCAAGTTGCTTTTC	FAM AGCATCCGTTCTACAGTGGGCCAAGA BHQ1
CD30	GATCAGCCACGGCTATATGGTGAG	AGCGGCAGGTTCTCCAAGGGTA	FAM CACACCAGCAGTCAATTCCTG BHQ1
OX40	AAGATGGCTCAGTGCAACTTCC	CAAGGTGGGTGGAGAGGGGCAA	FAM TCTGCAGATGTAGACCAGGCA BHQ1
BAFF	GAAGTGTGCCATGTGAGTTATGAGA	TCACCCAAGGCAAAAAGCA	FAM TCCTTTGCCAACACGCACCGC dark
BAFF-R	ACTTCAGAAGGAGTCCAGCAAGAG	CAGGTAGGAGCTGAGGCATGAG	FAM CCCTGGAAAATGTCTTTGTACCCTCCTCA BHQ1
APRIL	CGAGTCTGGGACACTGGAATTT	AGATACCACCTGACCCATTGTGA	FAM CTGCTCTATAGTCAGGTCCTGTTTCATGATGTGAC BHQ1
TACI	CATTCTGCCCCAAAGATCAGTAC	TGCTCTTTTCGGCAATTGATG	FAM CAGCCAGAGGAGCCAGCGCAC BHQ1
BCMA	TCCAACCCTCCTGCAACCT	CGTGTACGTCCCTTTCACTGAA	FAM TCAGCCTTACTGTGATCCAAGCGTGACC BHQ1
CCL19	CCTTCCGCTACCTTCTTAATGAAG,	ACAGAGCTGATAGCCCCTTAGTGT	FAM TGCAGGGTGCCTGC TAMRA
CCL21	TCCCGGCAATCCTGTTCT-C	TTCTGCACCCAGCCTTCCT	FAM CCCCGGAAGCACTCTAAGCCTGAGCTAT BHQ1
β2-mglob	CTGCAGAGTTAAGCATGCCAGTAT	ATCACATGTCTCGATCCCAGTAGA	CalFluor560/NED CGAGCCCAAGACC (BHQ1/MGB)
β-actin	CGTGAAAAGATGACCCAGATCA	TGGTACGACCAGAGGCATACAG	Yakima Yellow TCAACACCCCAGCCATGTACGTAGCC dark

*: All primers from MWG erofins (UK), probes from Applied Biosystems (UK)

**: CD3ε, CD19, CCR7, CXCR3, CXCR4, CXCR7, CXCL9, CXCL10, IL-10Rα TaqMan gene expression assay (Applied Biosystems UK)

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